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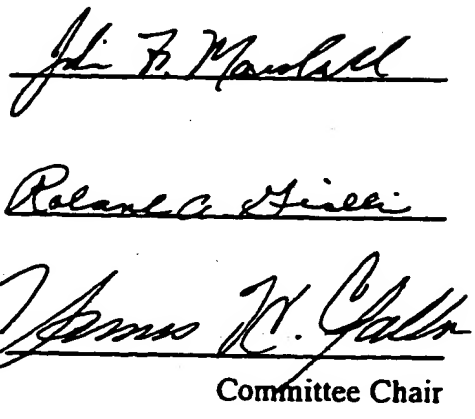

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1997

DEDICATION

To my parents,

Inzia Fay Willmon

&

James Ralph Reid,

who passed away

during my tenure

as a graduate student.

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ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my advisor, Dr. James Fallon, for his unending belief in my work and for allowing me the freedom to pursue my own research ideas.

I would also like to acknowledge Dr. Sandra Loughlin for her early support and understanding, and for stimulating my interest in neurotrophic factors and their roles in neuroregeneration. I would like to take this opportunity to credit her with guiding me to the topic of my research.

I would like to thank Dr. Richard Robertson for his support and leadership.

I would like to recognize Dr. Roland Giolli for his technical assistance with the histological studies, and Dr. Giolli, Dr. Robert Blanks and Dr. John Marshall for their stimulating scientific discussions and encouragement.

I would like to recognize the tireless efforts of the many undergraduate students, technicians, and volunteers who assisted in these studies. I especially appreciate the contributions of Janie Baratta to all stages of the research.

I would also like to thank the department administrative staff, particularly Kathleen Bisuano, Scott Talkovic and Linda Estacion, for their assistance.

I would like to express my appreciation for the numerous sources of financial support for the research: a UC Irvine Chancellor's Fellowship, an American Foundation for Aging Research Fellowship, NIH Developmental Neurobiology Predoctoral Traineeship NS07351-5, and a UC Irvine Committee of 1000 Graduate Research Award.

Finally, I would like to thank Gail Phillips for her love and support.

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ABSTRACT OF THE DISSERTATION

Transforming Growth Factor Alpha in the Nigrostriatal System and its Role in Regeneration of the Mammalian Forebrain

by

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Doctor of Philosophy in Biological Sciences

University of California, Irvine, 1996

Professor James H. Fallon, Chair

Dopamine neurons of the ventral mesencephalon suffer extensive losses during the course of human idiopathic Parkinson's disease (PD). Transforming growth factor alpha (TGFα), a neurotrophic factor from the epidermal growth factor (EGF) family, has been shown to support the survival of these dopamine cells and enhance several indices of their function. Recent studies have shown that TGFα is dramatically increased selectively in the human Parkinsonian striatum. The present series of *in situ* hybridization experiments were undertaken in a rodent model of PD. Specifically, the abundance of mRNA transcripts encoding TGFα precursor peptide and its receptor, the EGF receptor, were examined in animals receiving lesions and/or TGFα infusions.

Lesions reduced striatal TGFα mRNA from normal levels and eliminated ipsilateral midbrain EGF receptor mRNA expression, and forebrain TGFα

infusions increased EGF receptor in the striatal subependymal zone. Of particular interest, however, was a striking ridge of cells expressing abundant EGF receptor mRNA that appeared in the ipsilateral striata of animals receiving combined lesions and striatal TGFα infusions.

Through a series of characterization experiments, this ridge was shown to have migrated from the subependymal region and was found to express markers for cells recently identified as neural stem cells. These cells have been shown to give rise to new neurons, astrocytes, and oligodendrocytes under certain culture conditions. *In vivo* stimulation of neural stem cells in the mature brain represents a novel strategy for the repopulation of degenerated brain regions with new neurons derived from a patient's own brain. Such a strategy offers substantial advantages over the use of embryonic donor tissue from aborted fetuses and over a proposed technique to removed neural precursor cells, culture them, then re-implant them after their numbers have been sufficiently increased.

CHAPTER ONE

INTRODUCTION — THE STRIATUM AND THE NIGROSTRIATAL SYSTEM

Anatomy, Connectivity and Neurochemistry

The basal ganglia are comprised of the striatum, pallidum, substantia nigra, ventral tegmental area, subthalamic nucleus, and amygdala. The striatum contains dorsal and ventral components and each of these components is further subdivided into additional anatomical structures. In humans, the dorsal striatum consists of the caudate nucleus and the putamen. The C-shaped caudate follows the curve of the lateral ventricles. Its tail portions extend past the ends of the inferior horns and join the amygdala in each temporal lobe. The head of the caudate turns ventrally from the anterior end of the anterior horns and fuses with the putamen. Although largely anatomically distinct in the human brain, they are combined into a common structure, the caudate-putamen or caudoputamen, in rodents.

The ventral striatum is comprised of the nucleus accumbens, olfactory tubercle, and the associated striatal cell bridges. The pallidum includes the globus pallidus, entopeduncular nucleus, substantia nigra pars reticulata (SNr), and the ventral pallidum. The entopeduncular nucleus and SNr have very similar afferent and efferent connections. The ventral pallidum contains regions that have a mix of connections that are similar to both the globus pallidus and entopeduncular nucleus. The other part of the substantia nigra, the pars

compacta (SNc), includes dopamine neurons that span the substantia nigra-ventral tegmental area (SN-VTA), as well as dopamine cell clusters in the SNr. The circuitry of the basal ganglia is complex, but is very similar in both rats and humans (Fallon and Loughlin, 1987; Heimer and Alheid, 1991; Alheid and Heimer, 1996), making the rat a useful model for studying the connections, neurochemistry, pharmacology, function, and clinical correlates of this system in the mammalian brain.

The striatum, in concert with other nuclei of the basal ganglia, contribute to the regulation of movement and emotion. A number of diseases affecting the system or its innervation are associated with profoundly debilitating motor impairment, often accompanied by affective disorders. Before examining the injured striatum, it is necessary to review the connectivity and neurochemistry of the normal, intact system.

The caudate and putamen are the primary input nuclei of the basal ganglia and receive major excitatory projections from the cerebral cortex and the centromedian and intralaminar nuclei of the thalamus. Corticostriatal afferents are glutamatergic. Afferents from the thalamus are also thought to be glutamatergic. The substantia nigra pars compacta (SNc) provides dense dopaminergic input to the striatum via the nigrostriatal pathway (for review of this system in the rat, see Fallon and Loughlin, 1995). The ventral striatum and nucleus accumbens receive the bulk of their dopaminergic innervation from dopamine cells of the ventral tegmental area (VTA) in the ventromedial mesencephalon. Limbic afferents from the amygdala and serotonergic fibers from the midbrain or raphé also terminate in the ventral striatum.

The distributions of striatal afferents and their terminations are not simply uniform representations of their regions of origin. The striatum is organized into patches or striosomes embedded in a functionally and chemically distinct surrounding matrix. This organization was originally demonstrated using histochemistry for acetylcholinesterase (AChE), which selectively stains the matrix (Graybiel and Ragsdale, 1978). Since then, enzyme histochemistry, immunocytochemistry, *in situ* hybridization, receptor binding with radiolabeled ligands, anterograde degeneration and other methods have been used to identify many additional markers that are differentially distributed in the two compartments. Markers for the matrix include calbindin, somatostatin and dopamine uptake [^{3}H]mazindol binding) sites (Gerfen, 1985; Gerfen et al., 1985; Graybiel and Moratalla, 1989; Voorn et al., 1989). Striosomes can be identified by their higher relative abundance of enkephalin, 5'-nucleotidase activity with nigrostriatal lesions, tyrosine hydroxylase, mu opioid receptor binding, and substance P (Graybiel et al., 1981; Graybiel and Chesselet, 1984; Herkenham and Pert, 1981; Schoen and Graybiel, 1992). As might be expected, however, there are interspecific and developmental variations in many of these markers and some are useful only in certain regions of the striatum.

The heterogeneity of chemical markers is further complicated by the selective origin and termination of many striatal pathways in the patch or matrix compartments. For instance, afferents from motor, cingulate, somatosensory and visual areas of cortex terminate in the matrix (Gerfen, 1984; Donoghue and Herkenham, 1986). The bulk of the corticostriatal afferents from deep layer V

and layer VI of limbic cortex terminate in the patches while most from more superficial layer V and layers II and III provide input to the matrix (Gerfen, 1989). Afferents from the ventral tegmental area (VTA) and the dorsal tier of the SNc provide dopaminergic input to the matrix. The patches receive dopamine innervation from the ventral tier of the SNc and dopamine cell clusters in the SNr (Schoen and Graybiel, 1992). In the dorsal striatum, inputs from nuclei in the medial division of the thalamus terminate in the patches while afferents from the lateral division — including anterior and posterior intralaminar and rostral ventral tier nuclei — predominantly innervate matrix tissue (Ragsdale and Graybiel, 1991). In addition, amygdalostriatal fibers originating in the basolateral nucleus of the amygdala selectively innervate the patch compartment (Ragsdale and Graybiel, 1988).

Striatal efferents are also differentially distributed with respect to the patch-matrix organization. The striatonigral pathway, one of the two major pathways originating in the striatum, has been shown to be comprised of two distinct projections. Fibers arising from neurons in the patch compartment terminate around dopamine neurons in the ventral SNc and in dopamine cell clusters in the SNr. Matrix neurons give rise to topographically-arranged projections to the the SNr, including non-dopaminergic areas and dopamine neurons whose dendrites are located in the SNr (Gerfen, 1984; Gerfen, 1985; Gerfen et al., 1985; Gerfen and Young, 1988; Jiminez-Castellanos and Graybiel, 1989).

The other major efferent projection, the striatopallidal pathway, projects to the globus pallidus. It has not been shown to be distributed with respect to the patch-matrix organization; however, it is neurochemically distinct from the

striatonigral system. The majority of striatopallidal fibers express enkephalin and not dynorphin or substance P. In contrast, few striatonigral projections contain enkephalin, but most express dynorphin and substance P (Gerfen and Young, 1988). In primates, the two systems also differ in their anatomical regions of origin: striatopallidal efferents arise mainly from the putamen while striatonigral efferents originate primarily in the caudate (Parent et al., 1984).

In addition to the heterogeneous distribution of striatal connections, several morphologically and chemically distinct types of neurons are found in the striatum (Albin et al., 1989; Bolam, 1984; Carpenter, 1991; Groves, 1983). They are traditionally classified as either spiny or aspiny based on their dendritic morphology. There are two generally recognized types of spiny neurons in the striatum. They contain various combinations of GABA, substance P, enkephalin and dynorphin, but are predominantly GABAergic. The medium spiny neurons (spiny type I) are by far the most abundant, comprising 90 to 95 percent of all striatal neurons. They have smooth cell bodies and dense accumulations of spines on the distal portions of their dendrites. Their dendritic arborizations range to about 200 μm from the somata. Medium spiny neurons are the principle terminal targets for dopaminergic neurons in the SNc, which form synapses predominantly on the necks of the dendritic spines. Spiny type II neurons are much larger, with variable dendritic arbors extending up to about 600 μm from the soma.

Spiny neurons are the projection neurons of the striatum. Those in the matrix containing GABA and substance P project predominantly to the internal segment of the globus pallidus (GP_i) and the SNr. Spiny GABAergic matrix

neurons containing enkephalin, on the other hand, innervate the external segment of the globus pallidus (GP_e). Spiny neurons in the patch compartment send the majority of their efferents to the SNc (Albin et al., 1989). Striatal projection neurons of the two major efferent pathways can also be distinguished by their dopamine receptor subtypes. Substance P/dynorphin neurons projecting to the substantia nigra express predominantly D_1 dopamine receptors, while enkephalinergic striatopallidal neurons express mainly D_2 receptors. Neither receptor type, however, is expressed exclusively in either projection (Besson et al., 1989; Gerfen et al., 1990).

Three recognized types of aspiny neurons comprise the population of striatal interneurons (Albin et al., 1989; Carpenter, 1991; Groves, 1983). Altogether they make up ten percent or less of the total number of neurons of the striatum. Aspiny type I neurons are the most common of the three and have smooth dendrites in arbors slightly smaller than those of medium spiny neurons. They are largely GABAergic, but many contain somatostatin and neuropeptide Y. Aspiny type II neurons are distinguished by their large cell bodies and AChE and choline acetyltransferase (ChAT) staining. This cell type forms symmetric synapses with medium spiny neurons. Medium aspiny type III neurons are the least well characterized but are thought to contain GABA. There are probably additional chemically- and connectionally-defined subsets of these classes of neurons beyond the ones already identified.

Topography and Development

Experiments with anterograde and retrograde tracers in striatal projections of the mesencephalic dopamine system revealed precise topographies in adult rodents (Fallon and Moore, 1978). The dorsal striatum receives dopaminergic innervation from neurons in the ventral and intermediate SN and VTA. The ventral striatum and nucleus accumbens receive dopaminergic input from the dorsal VTA and intermediate SN (Fallon, 1988).

Neurogenetic gradients in the developing system parallel the topographic arrangements of projections in the mature system. The dorsolateral portion of the SN is the earliest produced in the embryo (before embryonic day 15 (E15) in the rat) (Altman and Bayer, 1981). Projections from this region innervate the lateral and ventral regions of the striatum (Carter and Fibiger, 1977; Beckstead et al., 1979; van der Kooy, 1979; Veening et al., 1980) which are also the earliest striatal areas generated (Bayer, 1984). As the striatum is populated with younger neurons in a ventrolateral-to-dorsomedial gradient, afferents arrive from more ventromedial (and later-produced) portions of the SN (generated after E15) to innervate these later-produced striatal areas. Thus, the youngest (ventromedial) nigral dopamine neurons innervate the youngest (dorsomedial) striatal neurons, and older (dorsolateral) nigral dopamine neurons innervate older (ventrolateral) striatal neurons. This pattern is repeated in the GABAergic striatonigral projections as well (Bunney and Aghajanian, 1976).

The neurons of the striatum are derived from a neuroepithelium surrounding the lateral ventricles in the prenatal and early postnatal brain. A ventricular zone initially lines the ventricles, later joined by the subventricular

(or subependymal) zone just deep to it. As the brain matures, the ventricular zone disappears, but the subependymal zone persists as a thin layer of cells. This zone has recently been shown to contain neural stem cells and progenitor cells which migrate along a defined, restricted path to replenish the labile interneuronal cell population in the olfactory bulb (Luskin 1993; Lois and Alvarez-Buylla, 1994).

Pathology

The striatum and its dopaminergic innervation are vulnerable to a number of conditions including several neurodegenerative diseases (Albin et al., 1989). For the purposes of the present discussion, particular attention will be paid to two of them: Huntington's disease and Parkinson's disease. Huntington's disease (HD) is an autosomal dominant hereditary disease (chromosome 4) characterized by progressive degeneration of the striatum, associated with involuntary choreathetotic movements of the limbs and face and disruptions of voluntary movement (Purdon et al., 1994 for review). Medium spiny GABAergic neurons in the matrix compartment are most affected, especially the GABA/enkephalinergic neurons projecting to the GP_e (Albin et al., 1989). Large aspiny cholinergic interneurons and small aspiny interneurons containing somatostatin, neuropeptide Y and NADPH-diaphorase, also found in the matrix compartment, are relatively spared (Ferrante et al., 1985; Ferrante et al., 1987; Kowall et al., 1987; Reiner et al., 1988). In more advanced stages of HD, however, neuronal degeneration includes all types of striatal neurons and

extends to other nuclei of the basal ganglia, the cerebral cortex, hypothalamus and cerebellum.

Parkinson's disease (PD) is characterized by resting tremor, rigidity, inability to initiate movement (akinesia) and slowness of movement (bradykinesia) (Marsden, 1990). The motor deficits are associated with progressive degeneration of the dopaminergic nigrostriatal pathway and, to various extents, loss of dopaminergic innervation to the nucleus accumbens, and degeneration of noradrenergic cells of the locus ceruleus and serotonergic neurons of the raphé (Javoy-Agid et al., 1984; Agid, 1991). Up to 80 percent of nigral dopamine neurons can be lost before significant motor deficits manifest themselves.

One of the major strategies for using peptides known as neurotrophic factors as therapeutic agents in the treatment of neurodegenerative diseases is to arrest the degenerative process and enhance the function of remaining cells. The studies presented in the following chapters will initially follow this approach, but will also expand the potential role of certain neurotrophic factors far beyond it.

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CHAPTER TWO

TRANSFORMING GROWTH FACTOR ALPHA AND THE EPIDERMAL GROWTH FACTOR FAMILY OF NEUROTROPHIC FACTORS

Neurotrophic factors are peptides that variously support the survival, proliferation, differentiation, size and function of nerve cells (for major review, see Loughlin and Fallon, 1993). While the numbers of identified trophic factors, or growth factors, are ever-increasing, most can be assigned to one or another established family based upon their structure or binding affinities. Growth factors from various families have been demonstrated to support dopaminergic neurons of the nigrostriatal system, the subject of the experiments described in the following chapters (Hefti, 1994; Unsicker, 1994 for reviews). Members of one such family, the epidermal growth factor (EGF) family, have been studied with increasing interest in recent years with the realization that they may play important roles in nigrostriatal development, maintenance and placticity.

EGF was the first of this family to be characterized (Savage and Cohen, 1972; Savage et al., 1972). Since then, additional members have been identified, including vaccinia virus growth factor (VGF) (Ventatesan et al., 1982), myxomavirus growth factor (MGF) (Upton et al., 1987), Shope fibroma virus growth factor (SFGF) (Chang et al., 1987), amphiregulin (AR) (Kimura et al., 1990), and heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama et al., 1991). A common feature of these factors is an amino acid

sequence containing six cysteines which form three disulfide cross links and support a conserved structure. This conserved structure underlies their common ability to bind the EGF receptor.

EGF is by far the most-studied of the family and was the first localized to brain tissue: EGF-like immunoreactivity (IR) was found in areas of developing and adult forebrain and midbrain including the globus pallidus, ventral pallidum, entopeduncular nucleus, substantia nigra, and the Islands of Calleja (Fallon et al., 1984). EGF mRNA was detected in the central nervous system (CNS) using dot blot hybridization on whole adult rodent brain RNA (Rall et al., 1985) and in numerous specific brain regions, including the striatum at low concentration, in a quantitative nuclease protection assay (Lazar and Blum, 1992).

Another member of the EGF family, transforming growth factor alpha (TGFα), has also been localized to brain tissue. It binds the EGF receptor (Todaro et al., 1980) and stimulates the receptor's tyrosine kinase activity (Reynolds et al., 1981). It elicits similar mitogenic responses in a wide variety of cell types (Derynck, 1992 for review). TGFα-IR has previously been shown to be heterogeneously distributed in neuronal perikarya throughout the adult rat CNS and in subpopulations of forebrain astrocytes (Code et al., 1987; Kudlow et al., 1989; Fallon et al., 1988; Fallon et al., 1990). TGFα mRNA was detected in whole rodent brain (Lee et al., 1985; Kudlow et al., 1989) and in striatum and other brain regions by a nuclease protection assay (Lazar and Blum, 1992;

Weickert and Blum, 1995) and by *in situ* nuclei acid hybridization (Wilcox and Derynck, 1988; Seroogy et al., 1993).

TGFα and EGF mRNAs reached their highest relative abundance (compared to total RNA) in the early postnatal period and decreased thereafter, suggesting a role in development (Lee et al., 1985; Kudlow et al., 1989; Lazar and Blum, 1992). In whole brain, the reduction was over 50 percent (Lazar and Blum, 1992); whereas, in striatum, relative TGFα mRNA dropped by two-thirds from peak levels (Weickert and Blum, 1995). At all developmental stages examined, whole brain TGFα mRNA exceeded EGF mRNA levels by more than an order of magnitude (Lazar and Blum, 1992). In the adult striatum, TGFα mRNA persisted at concentrations more than two orders of magnitude higher than EGF mRNA (Lazar and Blum, 1992). The far greater abundance of TGFα mRNA than EGF mRNA suggests that TGFα may function as the primary ligand for the EGF receptor in the developing and adult nigrostriatal system.

The EGF receptor was localized to astrocytes and subpopulations of cortical and cerebellar neurons in rat brain and in neurons in human autopsy brain specimens using immunocytochemistry (Gómez-Pinilla et al., 1988; Werner et al., 1988). EGF binding sites were revealed in rat cortical and subcortical areas, including striatum, in an autoradiography study with radiolabeled EGF (Quirion et al., 1988). *In situ* hybridization studies demonstrated EGF receptor mRNA in striatum and cells of the ventral mesencephalon (Seroogy et al., 1994) and in proliferative regions in developing

and adult rat brain (Seroogy et al., 1995). As with relative EGF and TGFα mRNAs, EGF receptor mRNA is most abundant in striatum and ventral midbrain early in development, and gradually declines as the animal matures (Seroogy et al., 1994).

Physiologically, TGFα acts on numerous cell types throughout the body, including many of neural origin (review, Derynck, 1992). It supports the survival of cultured central neurons (Morrison et al., 1987; Zhang et al., 1990) and, unlike EGF, enhances survival and neurite outgrowth of dorsal root ganglion sensory neurons (Chalazonitis et al., 1992). It also stimulates proliferation and differentiation of neuronal and glial progenitors cells from developing and adult brains (Anchan et al., 1991; Reynolds and Weiss, 1992; Reynolds et al., 1992).

The trophic effects of EGF-family peptides on mesencephalic dopaminergic neurons in culture have also been studied in recent years. EGF enhances the survival of E16 dopamine neurons in mixed midbrain cultures (Casper et al., 1991), but its stimulation of their dopamine uptake is modest (Knusel et al., 1990). TGFα also supports the survival of mesencephalic dopamine neurons in dissociated cell culture, but its effect is more selective than that of EGF (Ferrari et al., 1991; Alexi and Hefti, 1993). Furthermore, TGFα stimulates dopamine uptake by dopamine neurons to a greater extent than EGF and enhances their neurite length, branch number, and soma size (Alexi and Hefti, 1993).

An additional important action of EGF-family growth factors is their ability to protect midbrain dopamine cells from neurotoxic assaults. This feature is especially important if the growth factors are to be used as a prophylactic agents to halt neurodegenerative processes. EGF has been shown to protect dopamine neurons from glutamate or quisqualate excitotoxicity in dissociated cell culture (Casper and Blum, 1995). It has also been demonstrated to protect cultured dopamine cells from the selective dopamine neurotoxin, 1-methyl-4-phenylpyridinium (MPP+) (Park et al., 1992) and increase dopamine uptake in MPP+ treated cultures (Hadjiconstantinou et al., 1991).

Studies with EGF *in vivo* yielded similar results. Intracerebroventricular (ICV) infusions of EGF reduced amphetamine-induced rotations, increased the numbers of surviving tyrosine-hydroxylase immunoreactive (TH-IR) cells in the SN, and increased striatal TH-IR fibers after transection of the nigrostriatal pathway in a rat model of Parkinson's disease (PD) (Pezzoli et al., 1991; Ventrella, 1993). ICV infusions of EGF into the brains of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mice enhanced dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) content and tyrosine hydroxylase activity in the striatum (Hadjiconstantinou et al., 1991; Schneider et al., 1995).

Despite its more potent activity than EGF *in vitro*, the trophic effects of TGFα *in vivo* — particularly in lesioned animals — have been largely unstudied. In the next chapter, the effects of infused TGFα on cells of the normal or lesioned nigrostriatal system are examined.

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CHAPTER THREE

MODULATION OF TGFα AND EGF RECEPTOR mRNA EXPRESSION BY 6-HYDROXYDOPAMINE LESION AND STRIATAL TGFα INFUSION

Introduction

Messenger RNAs for transforming growth factor (TGFα) and its receptor, the epidermal growth factor (EGF) receptor, have been localized in the developing and adult nigrostriatal system in a pattern consistent with the peptide's putative role as a target-derived growth factor for nigrostriatal dopaminergic neurons (Fallon et al., 1990; Fallon and Loughlin, 1995; Lazar and Blum, 1992; Seroogy et al., 1993; Seroogy et al., 1994; Wilcox and Derynck, 1988; and see Appendix for studies related to the present research).

In addition to its distribution, physiological experiments demonstrate that TGFα has potent trophic effects on dopamine neurons and numerous other cell types throughout the body, including many of neural origin (review, Derynck, 1992). It supports the survival of cultured central neurons (Zhang et al., 1990) and, unlike EGF, enhances survival and neurite outgrowth of dorsal root ganglion sensory neurons (Chalazonitis et al., 1992). It also stimulates proliferation and differentiation of neuronal and glial progenitors cells from developing and adult brains (Anchan et al., 1991; Reynolds and Weiss, 1992; Reynolds et al., 1992).

TGFα has been shown to have potent trophic effects specifically in the nigrostriatal system. The peptide selectively supports the survival of mesencephalic dopamine neurons in dissociated cell culture (Alexi and Hefti, 1993). Under similar conditions, EGF has much less selective trophic influences in midbrain cultures (Ferrari et al., 1991). Furthermore, TGFα stimulates dopamine uptake by dopamine neurons to a greater extent than EGF and enhances their neurite length, branch number, and soma size (Alexi and Hefti, 1993). Various markers of intact nigrostriatal innervation or indicators of protection against dopamine neurotoxins are enhanced by TGFα or EGF *in vitro* (Casper et al., 1991; Park et al., 1992; Casper and Blum, 1995) and *in vivo* (Hadjiconstantinou et al., 1991; Pezzoli et al., 1991; Ventrella et al., 1993; Schneider and DiStefano, 1995).

Therapeutic administration of neurotrophic factors from the EGF family and others has been suggested as a possible treatment strategy for idiopathic Parkinson's disease (PD) (Hefti, 1994; Unsicker, 1994). In PD, nigral dopamine neurons are lost selectively, resulting in depleted dopaminergic innervation of the striatum (Javoy-Agid et al., 1984). TGFα and EGF (and other) neurotrophic factors are increased in the striata but not in other brain regions of PD patients, possibly as a compensatory response to the loss of dopamine neurons (Mogi et al., 1994). However, an autoradiographic study showed no change in midbrain EGF binding sites in PD patients compared to normal controls (Villares et al., 1993). In the present study, we used *in situ* hybridization to determine whether nigrostriatal TGFα or EGF receptor mRNA expression was altered by

intrastriatal infusion of TGFα. Further, we examined the influence of unilateral 6-OHDA lesions on their expression in infused and uninfused animals.

Materials and Methods

Animals. Adult male adult Sprague-Dawley rats (250-350 g) were obtained from Simonsen (Gilroy, CA) and assigned to one of five treatment groups: (1) striatal TGFα infusion, nigral 6-OHDA lesion; (2) TGFα infusion, no lesion; (3) artificial cerebrospinal (aCSF) infusion, lesion; (4) aCSF infusion, no lesion; (5) no infusion, no lesion. Four to eight animals were used per experimental group. The animals were monitored after each surgical procedure until fully recovered and maintained at all other times in a temperature and humidity controlled campus vivarium. Use of the animals and the experimental procedures employed were approved by the University of California, Irvine, Animal Research Committee in accordance with National Institutes of Health guidelines.

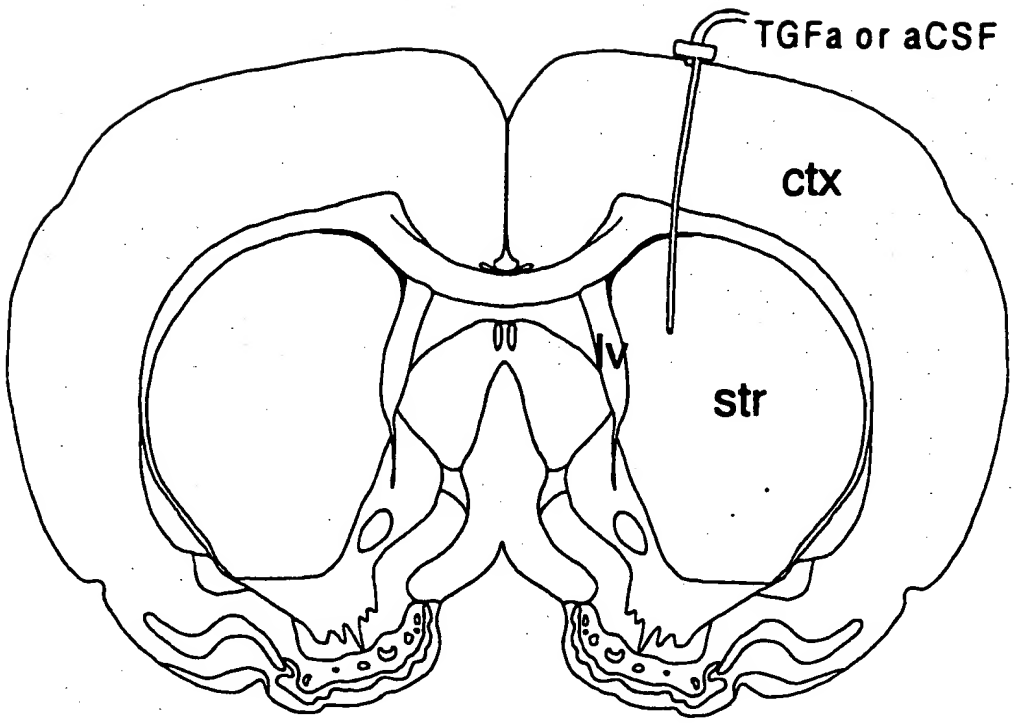
6-hydroxydopamine lesions. Rats were anesthetized with 8 mg xylazine and 100 mg ketamine per kilogram body weight. A chilled solution of 4.8 mg/ml 6-hydroxydopamine HCl (6-OHDA) (Sigma, Inc.) in 0.9% saline with 0.01% ascorbic acid was prepared immediately before injection. Using sterile technique, an 8 µl volume was stereotaxically injected into the left substantia nigra (+3.7 A/P; +2.1 M/L; +2.0 D/V) at a rate of 1 µl min^{-1} using interaural zero as a reference (Paxinos and Watson, 1986). The success and extent of 6-OHDA lesions were monitored by tyrosine hydroxylase mRNA *in situ* hybridization in the midbrain. Tyrosine hydroxylase (TH) is the rate-limiting

enzyme in the dopamine synthetic pathway and is a common marker for dopamine-producing neurons. One animal with an incomplete lesion (retaining significant numbers of nigral TH-IR cells) was excluded from the study and is not included in the total number of animals.

Infusions. Osmotic minipumps (model 2002, Alzet, Inc.) were implanted four to five weeks post lesion. The minipumps were filled with approximately 200 µl of either 0.05 µg μl^{-1} transforming growth factor alpha (TGFα) (courtesy Drs. D. Twardzik and J. Ranchalis, Bristol-Myers Squibb) in artificial cerebrospinal fluid (aCSF) for experimental animals, or aCSF only for control animals, and incubated overnight at 37°C prior to implantation. Following anesthesia as above, and under sterile conditions, the 5 mm cannula attached to the minipump (brain infusion kit, Alzet, Inc.) was stereotaxically implanted into the left caudate-putamen (+1.2 A/P; +2.7 M/L) using Bregma as a reference (Paxinos and Watson, 1986) and fixed to the skull with carboxylate cement (Figure 3.1). The minipump itself was placed subcutaneously in the interscapular region. The infusate was delivered directly into the striatum over a period of two weeks at a rate of 0.5 µl h^{-1}.

Tissue preparation. At the end of the infusion period, animals were sacrificed by decapitation. Their brains were quickly removed, frozen in isopentane at -20°C and stored at -70°C. Coronal cryostat sections were cut at 20 µm and thaw-adhered to Vectabond (Vector Labs, Inc.) coated slides in ordered anterior-to-posterior rows. The sections were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for one hour, rinsed in

Figure 3.1. Schematic of a coronal section of rat forebrain depicting the location of the infusion. Transforming growth factor alpha (TGFα) or artificial cerebrospinal fluid (aSCF) was administered continuously over a two week period from an Alzet osmotic minipump. The infusate was delivered directly into the striatum via a 5 mm stainless steel cannula fixed to the top of the skull with dental cement. (str, striatum; ctx, cerebral cortex; lv, lateral ventricle).


TGFa or aCSF
ctx
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phosphate buffer and air dried. Sections were stored with desiccant at -20°C until processed.

Hybridization probes. TGFα mRNA probes were generated from a 550 nucleotide XbaI/BamHI cDNA fragment from the 5' end of rat TGFα (kindly provided by Drs. M.S. Kobrin and J.M. Korc, University of California, Irvine) subcloned into pGEM 7Zf (Promega, Inc.). Antisense and sense probes were transcribed with SP6 and T7 polymerases, respectively. Rat EGF receptor mRNA probes were produced from a 718 base pair BamHI/SphI insert from the 5' end of the gene (also courtesy Drs. M.S. Kobrin and J.M. Korc, U.C. Irvine) in pGEM 7Zf. Probes for rat TH were created using the 1.2 kb BamHI/EcoRI fragment subcloned into pGEM 7Zf. Antisense subclones for EGF receptor and TH were transcribed with T7 polymerase. Sense subclones for EGF receptor and TH were transcribed with SP6 polymerase. All probes were radiolabeled by transcription in the presence of [^{35}S]UTP (NEN Research Products, Inc.).

In situ *hybridization. In situ* nucleic acid hybridization was performed according to the method described by Simmons et al. (1989). Parallel sections from experimental and control animals were hybridized overnight at 65°C with sense or antisense probes at a concentration of 10^7 c.p.m./ml. Adjacent sections from the same animals were hybridized to each of the probes so that direct comparison could be made of their anatomical distributions.

Slides from experimental and control animals were grouped together and apposed with ^{14}C-labeled brain paste standards to autoradiographic BetaMax Hyperfilm (Amersham, Inc.) for three to seven days. After successful development of the autoradiography film, the slides were dipped in Kodak

NTB-2 emulsion and exposed for four weeks. The autoradiographic sheet film and NTB-2 emulsion were developed with D-19 developer and Rapid Fix (Kodak, Inc.). The brain sections were then counterstained with thionin and coverslipped.

Analysis and quantitation. Dipped and stained sections were examined and photographed under bright and dark field microscopy. Autoradiograms were analyzed quantitatively using an MCID system (Microcomputer Imaging Device, Imaging Research, Inc.). Densitometry readings were sampled at multiple sites within each anatomical region of interest and averaged. Relative concentrations of TGFα and EGF receptor hybridization were then estimated using a computer-generated third degree polynomial standard curve constructed from the ^{14}C brain paste standards. The estimated values for each region in each treatment group were then averaged and their standard errors calculated. Brain regions ipsilateral to the experimental treatments were compared to the corresponding contralateral regions in the same sections or to corresponding regions in control brains at approximately the same positions. Significance of the comparisons was determined using the Student's *t*-test.

Results

Normal expression and control infusions. Expression of TH, TGFα and EGF receptor mRNAs in the striatum, the striatal subependymal region, and the substantia nigra in control animals receiving striatal infusions of aCSF was indistinguishable from that in normal animals (see Appendix for normal developing and adult expression). TH mRNA hybridization was prominent

Figure 3.2. Photomicrographs of a coronal sections through anterior (B), middle (C) and posterior (D) adult rat mesencephalon showing *in situ* hybridization localization of tyrosine hydroxylase mRNA. A control section hybridized with a sense probe for TH mRNA is shown (A) for comparison. TH mRNA hybridization was intense throughout the substantia nigra (sn) and ventral tegmental area (vta). Scale bar = 5 mm.

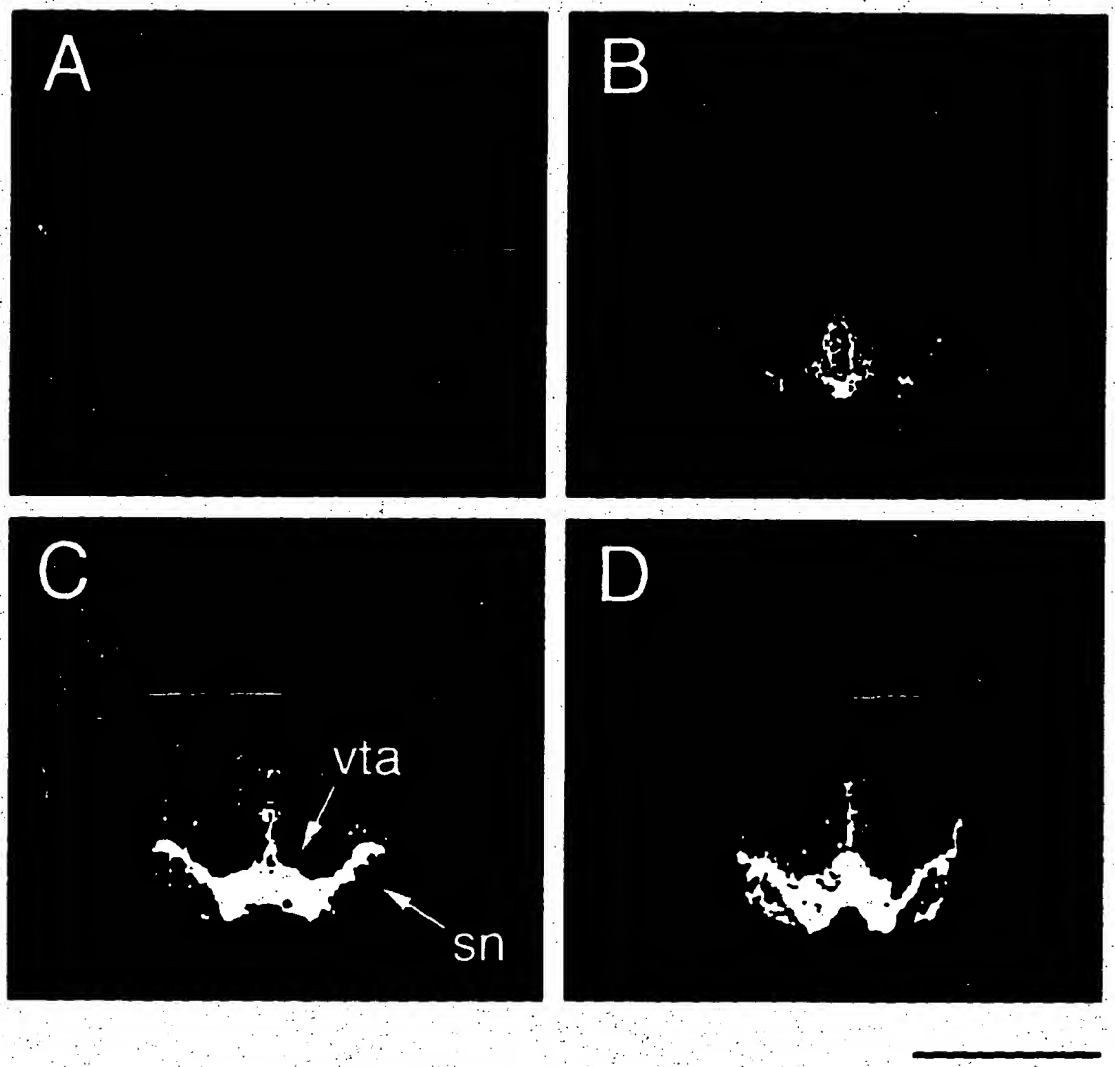
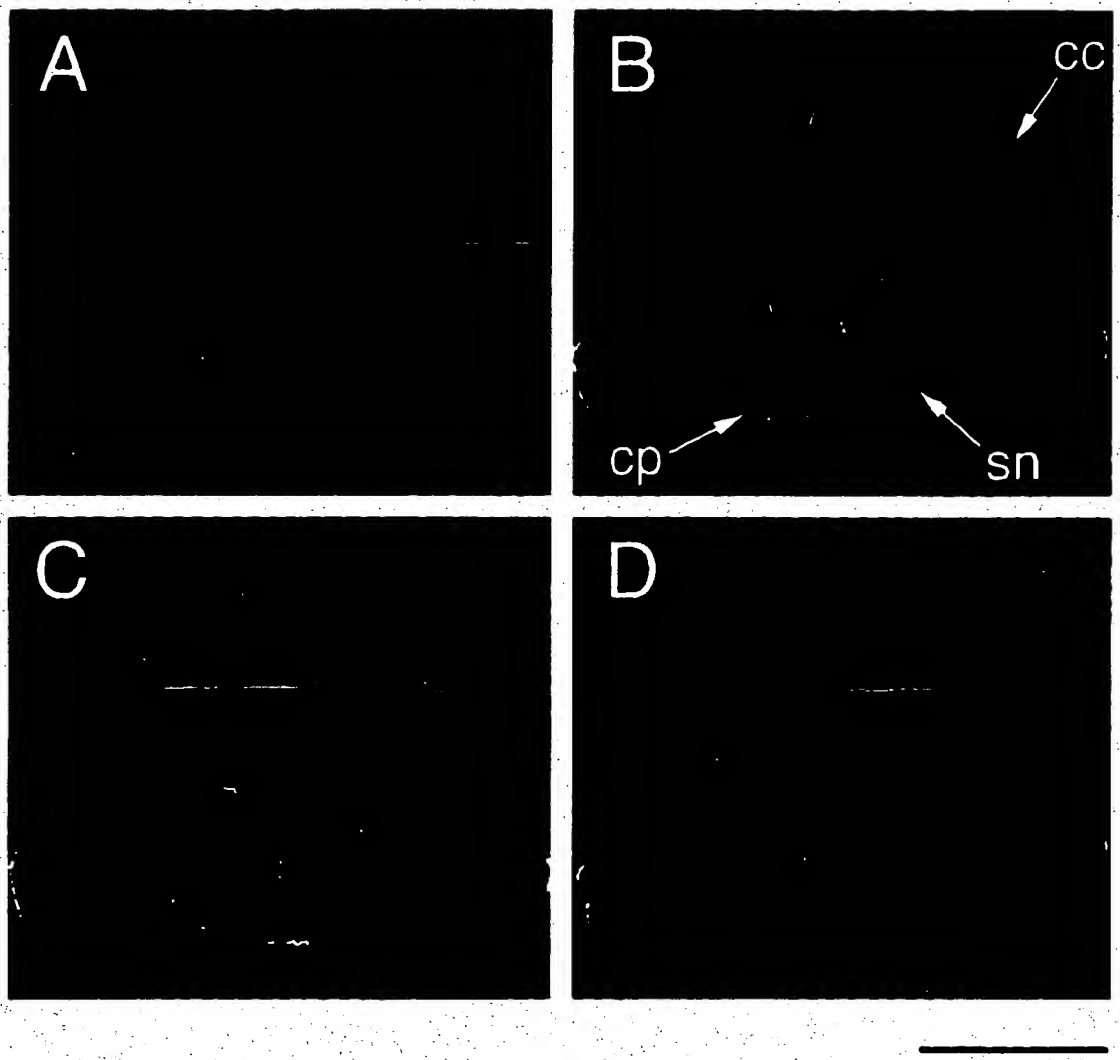

A
B
C
vta
sn
D

Figure 3.3. Photomicrographs of coronal sections through anterior (A), middle (B) and posterior (C) adult rat mesencephalon showing *in situ* hybridization localization of TGFα mRNA. A control section hybridized with sense probe for TGFα mRNA is shown for comparison (A). Moderate hybridization was localized to callosal fiber tracts underlying the cortex (cc) and to the cerebral peduncle (cp), but was not found above background levels in the substantia nigra (sn). Scale bar = 5 mm.


A
B
cc
cp
sn
C
D

throughout the SN-VTA (Figure 3.2). TGFα mRNA was not detected in the substantia nigra (Figure 3.3). EGF receptor mRNA, however, was prominent in the medial substantia nigra pars compacta (SNc), the paranigral, parabranchial, and interpeduncular nuclei of the ventral tegmental area (VTA) (Figure 3.4).

TGFα mRNA hybridization was expressed in the caudate-putamen and nucleus accumbens (NA), being slightly less dense in the NA (Figure 3.5). EGF receptor hybridization was found at low levels throughout the body of the striatum and NA with higher punctate expression dispersed throughout the low level background, and at moderate levels in the proliferative regions of striatum bordering the lateral ventricles (Figure 3.6).

Effects of 6-OHDA lesions. Unilateral nigral 6-OHDA lesions reduced TGFα mRNA hybridization in the ipsilateral striatum by 25 percent, but had no effect on contralateral TGFα mRNA hybridization (Figures 3.7 and 3.16). Striatal and subependymal EGF receptor hybridization were unchanged in lesioned animals compared to normals (Figures 3.8, 3.17 and 3.18). In the midbrain, 6-OHDA lesions abolished EGF receptor hybridization in the ipsilateral SN-VTA (Figure 3.9).

Effects of TGFα infusions. In unlesioned animals receiving TGFα infusions, TGFα mRNA hybridization in the infused striatum was unchanged compared to the contralateral striatum or striata from normal animals (Figures 3.10 and 3.16). A few of the animals receiving infusions of either TGFα or aCSF displayed a slight increase of TGFα mRNA hybridization immediately around

Figure 3.4. Photomicrographs of EGF receptor mRNA hybridization in an anterior-to-posterior series of adult rat coronal sections through the mesencephalon. A sense-probed control section is shown for comparison (A). In sections through the substantia nigra (sn) (B through D), hybridization is moderately abundant in the hippocampus (hip), the medial portion of the substantia nigra and the parabranchial and paranigral nuclei of the ventral tegmental area (vta). In the most-caudal midbrain (D), the interpeduncular nucleus (ip) was the most intensely hybridized. Scale bar = 5 mm.

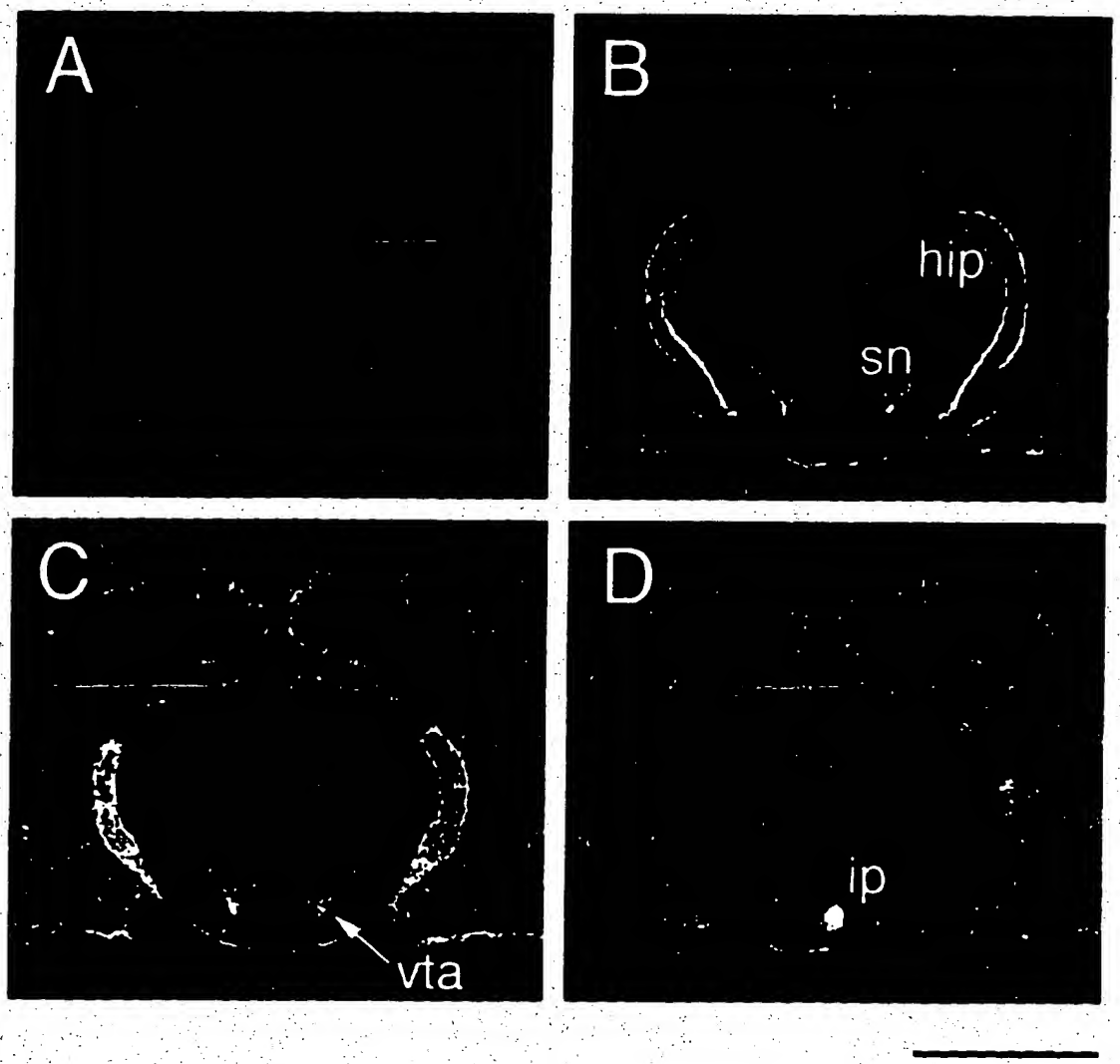

A
B
hip
sn
C
vta
D
ip

Figure 3.5. Photomicrographs of TGFα mRNA *in situ* hybridization to an anterior-to-posterior series of coronal sections (B to D) through the forebrain in an adult rat receiving a control infusion of aCSF. A control section hybridized with sense probe is shown for comparison (A). Hybridization was most prominent in the caudate-putamen (cp), nucleus accumbens (na) and olfactory tubercle (ot). Slightly less-intense hybridization was seen in the corpus callosum (cc). Scale bar = 5 mm.

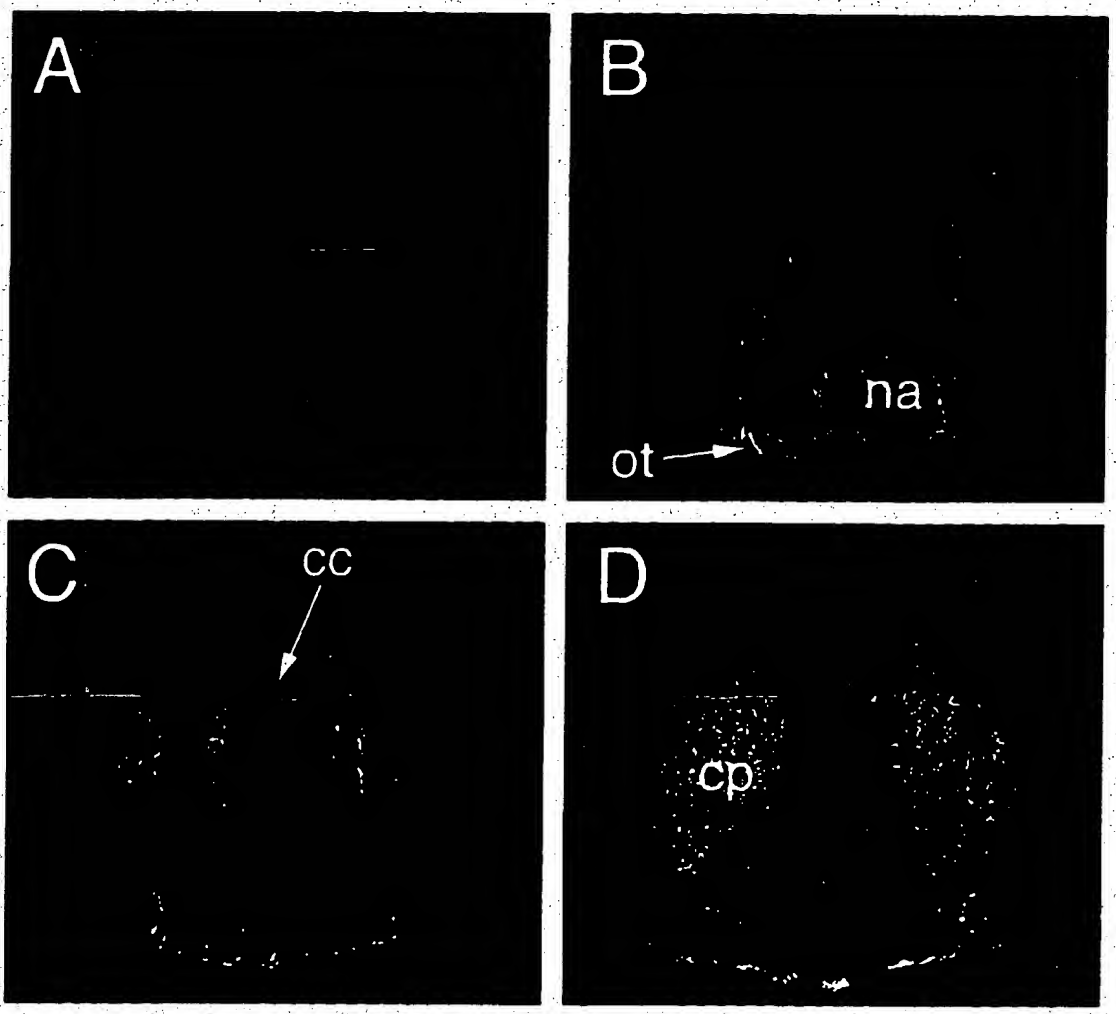

A
B
na
ot
C
cc
D
cp

Figure 3.6. Photomicrographs of EGF receptor mRNA *in situ* hybridization to an anterior-to-posterior series of coronal sections (B to D) through the forebrain in an adult rat receiving a control infusion of aCSF. A control section hybridized with a sense probe is shown for comparison (A). In the striatum, hybridization was dispersed punctately throughout the body of the caudate-putamen (cp) and intensely along the striatal border with the lateral ventricles. Scale bar = 5 mm.

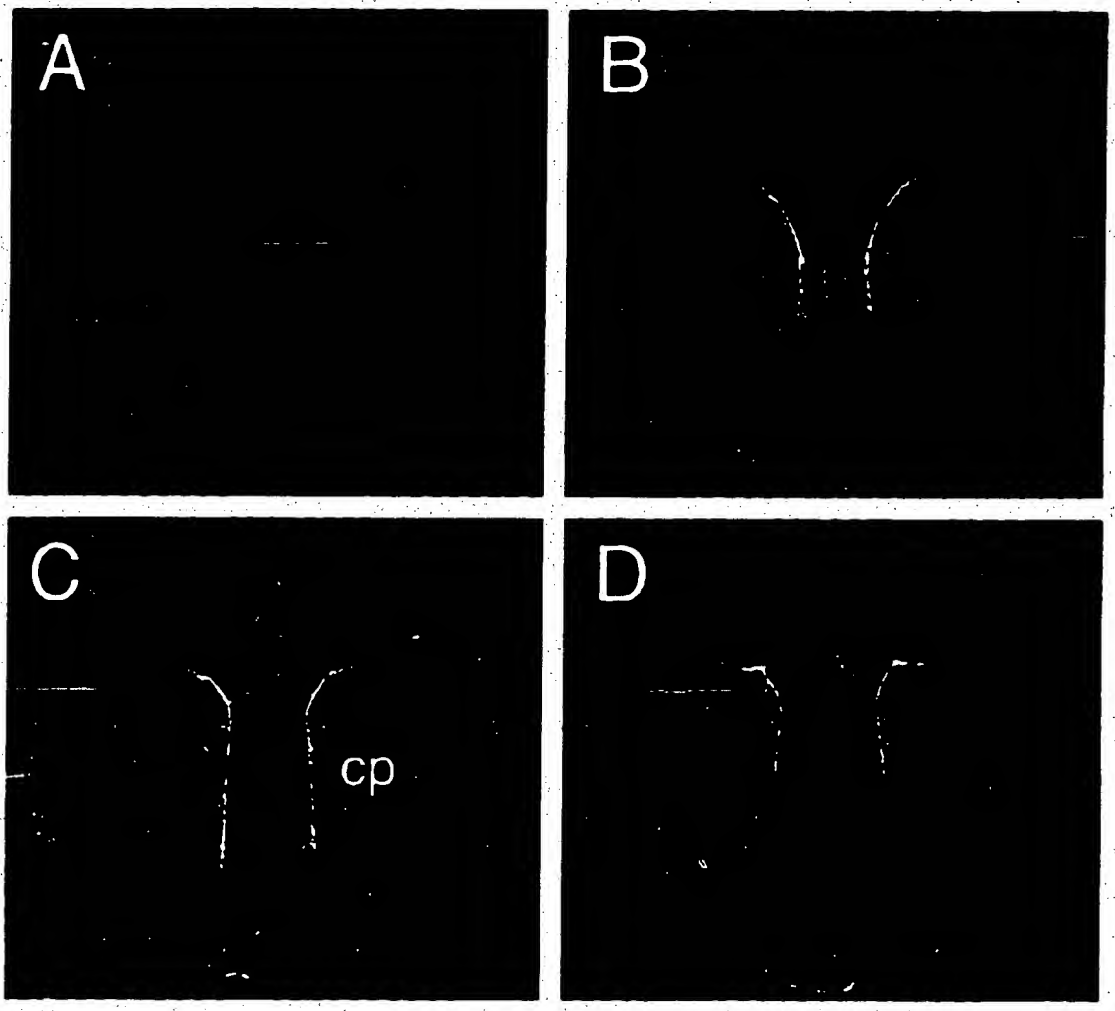

A
B
C
cp
D

Figure 3.7. Photomicrograph of TGFα mRNA *in situ* hybridization to a coronal section through the striatum in an adult rat receiving a control infusion of aCSF and an ipsilateral nigral lesion with 6-OHDA. Hybridization density was reduced in the caudate-putamen ipsilateral to the lesion. The infusion cannula implantation site can be seen in the ipsilateral striatum (arrowhead).

Figure 3.8. Photomicrograph of EGF receptor mRNA *in situ* hybridization to a coronal section through the striatum in an adult rat receiving a control infusion of aCSF and an ipsilateral nigral lesion with 6-OHDA. Hybridization density in the ipsilateral striatum was unchanged from the contralateral side. The infusion cannula scar can be seen in the ipsilateral striatum (arrowheads).

Figure 3.9. Photomicrograph of EGF receptor mRNA *in situ* hybridization to a coronal section through the midbrain in an adult rat receiving a control infusion of aCSF and an ipsilateral nigral lesion with 6-OHDA. Hybridization density in the ipsilateral substantia nigra (arrowhead) was reduced to background levels.

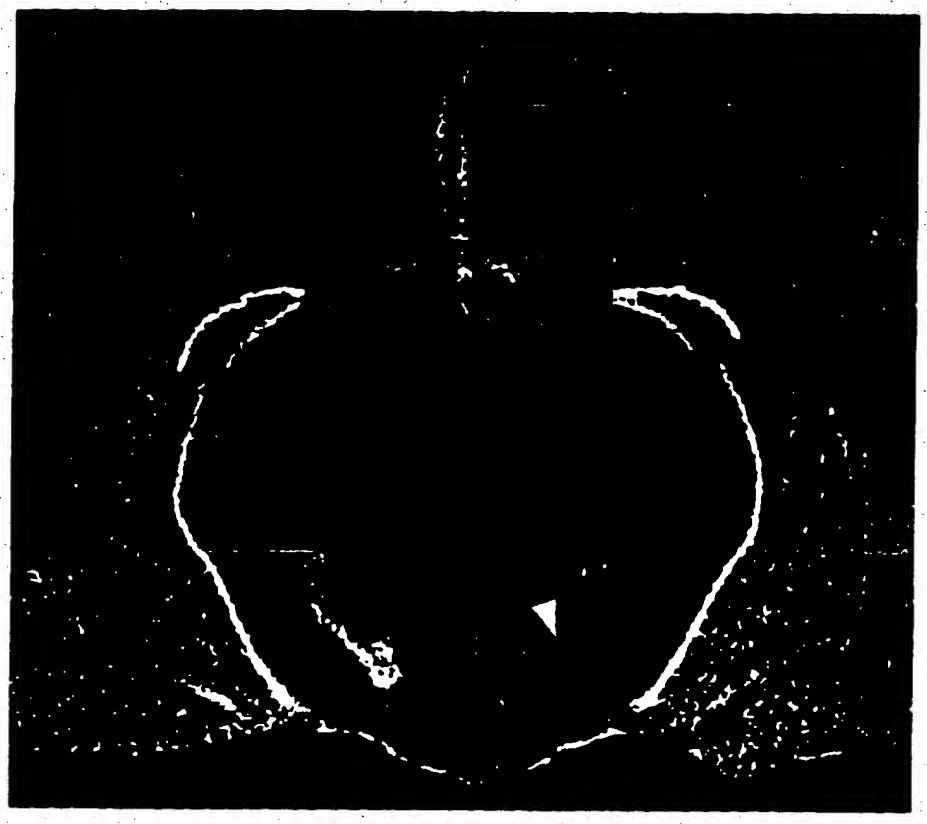


Figure 3.10. Photomicrograph of TGFα mRNA *in situ* hybridization to a coronal section through the striatum in an adult rat receiving an infusion of TGFα peptide. Hybridization density in the ipsilateral caudate-putamen was not significantly affected compared to the contralateral striatum. The infusion cannula scar can be seen in the striatum infused with the peptide (arrowhead).

the infusion cannula scar (data not shown). TGFα mRNA hybridization in the substantia nigra was not increased to detectable levels by the infusions (data not shown).

Hybridization to EGF receptor mRNA was dramatically increased in the ipsilateral subependymal region, but not in the rest of striatum, in all animals receiving TGFα infusions (Figures 3.11, 3.17 and 3.18). No change from normal was observed in EGF receptor hybridization in the SNc with TGFα infusion alone (Figure 3.12).

Combined TGFα infusion and 6-OHDA lesion. Striatal TGFα mRNA hybridization in animals receiving both striatal TGFα infusions and subsequent nigral 6-OHDA lesions was indistinguishable from that in lesion-only animals (Figures 3.13 and 3.16). Similarly, EGF receptor hybridization in the midbrains of TGFα-infused/6-OHDA-lesioned animals was indistinguishable from that in lesion-only animals (Figure 3.14).

EGF receptor mRNA hybridization in the forebrain revealed an anomolous ridge of dense hybridization in the body of the ipsilateral striatum in addition to the increased hybridization in the subependymal region (Figures 3.15, 3.18 and 3.19). The ridge was found in five of the six rats in the combined TGFα infusion/lesion group, but only in one of six rats in the TGFα infusion/nonlesion group. EGF receptor hybridization in the surrounding striatum was unchanged (Figure 3.17).

Figure 3.11. Photomicrograph of EGF receptor mRNA *in situ* hybridization to a coronal section through the striatum in an adult rat receiving an infusion of TGFα peptide. Note the dramatic increase in hybridization density in the medial striatum adjacent to the lateral ventricle compared to the contralateral striatum. The infusion scar is not evident in this section.

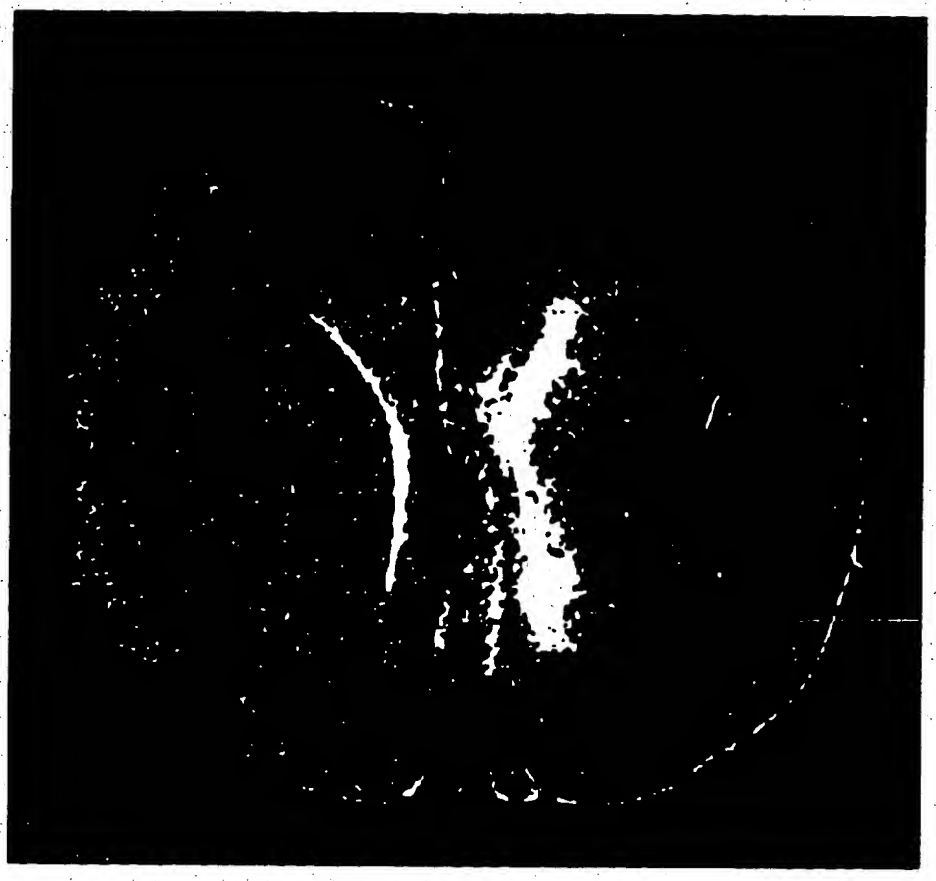


Figure 3.12. Photomicrograph of EGF receptor mRNA *in situ* hybridization to a coronal section through the midbrain in an adult rat receiving striatal infusion of TGFα peptide. Hybridization in the ventral midbrain was not obviously different from that in normal control animals.

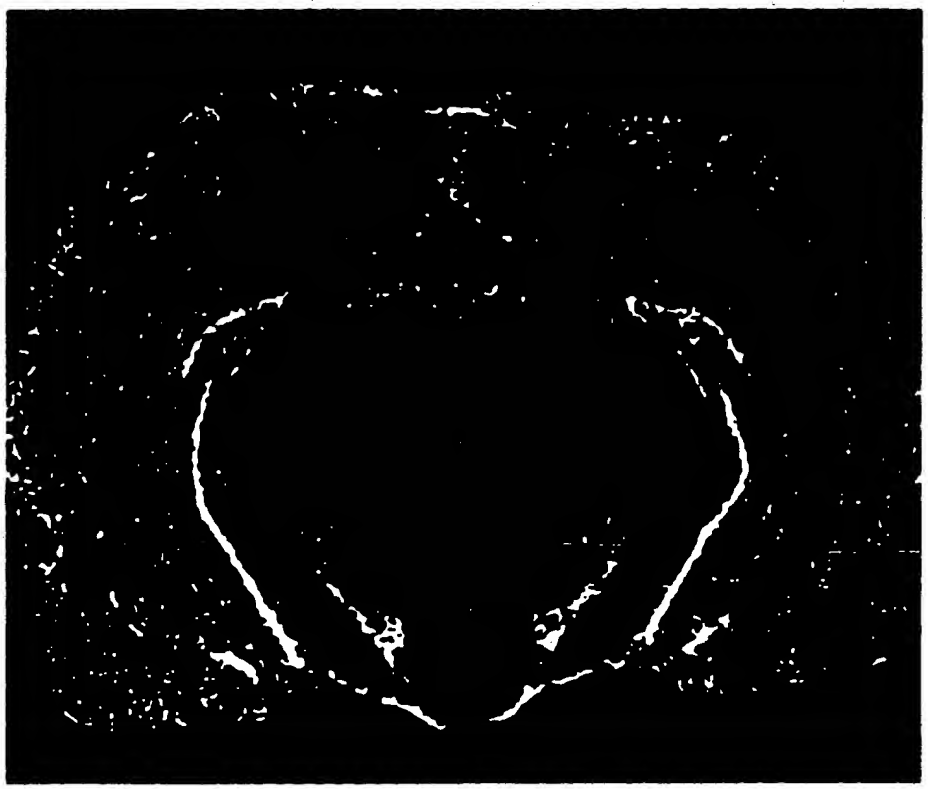


Figure 3.13. Photomicrograph of TGFα mRNA *in situ* hybridization to a coronal section through the forebrain in an adult rat receiving both striatal infusion of TGFα peptide and nigral lesion with 6-OHDA. Hybridization density in the ipsilateral caudate-putamen was reduced as in the lesion-only animals. Arrowheads denote the location of the infusion cannula scar.

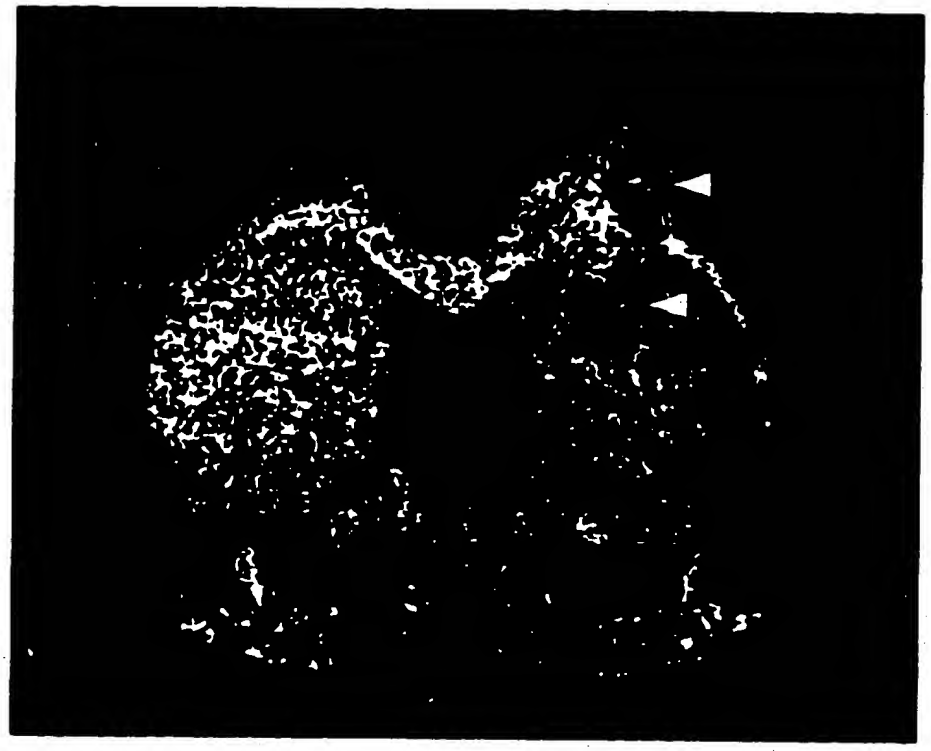


Figure 3.14. Photomicrograph of EGF receptor mRNA *in situ* hybridization to a coronal section through the mesencephalon in an adult rat receiving both striatal infusion of TGFα peptide and nigral lesion with 6-OHDA. Hybridization density in the ventral midbrain ipsilateral to the lesion and infusion was reduced to background levels as in the lesion-only animals.

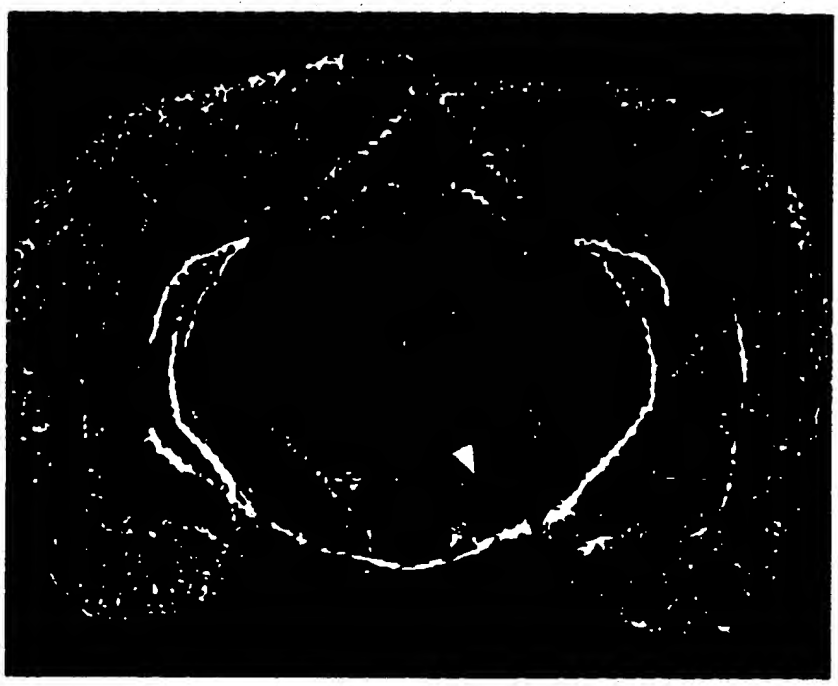


Figure 3.15. Photomicrograph of EGF receptor mRNA *in situ* hybridization to a coronal section through the forebrain in an adult rat. Five of six animals receiving both striatal infusions of TGFα peptide and nigral lesions with 6-OHDA exhibited this pattern of hybridization. One of the six animals with TGFα infusions but without a nigral lesion displayed the pattern. The overwhelming feature was the ridge of intense EGF receptor hybridization well into the body of the striatum. Hybridization density along the ipsilateral ventricle was also markedly increased compared to the contralateral side. Hybridization in the non-ridge portions of the body of the striatum was unchanged from that in either the contralateral striatum or striata in aCSF-infused control animals.

Figure 3.16. Bar graph showing the average standardized densities of TGFα mRNA hybridization in striata in each of the five groups examined. White bars represent averages from the ipsilateral striata. Filled bars denote averages from contralateral striata. Average hybridization density was significantly reduced by one-quarter ipsilateral to the treatments in both of the groups receiving nigral 6-OHDA lesions. The striatal infusion of TGFα peptide had no impact on the decrease. Averages ± S.E.M. (Student's *t*-test, paired for ipsilateral-contralateral comparisons; P values, * $p < 0.005$, ** $p < 0.001$).

TGFα mRNA Hybridization Densities in the Striatum

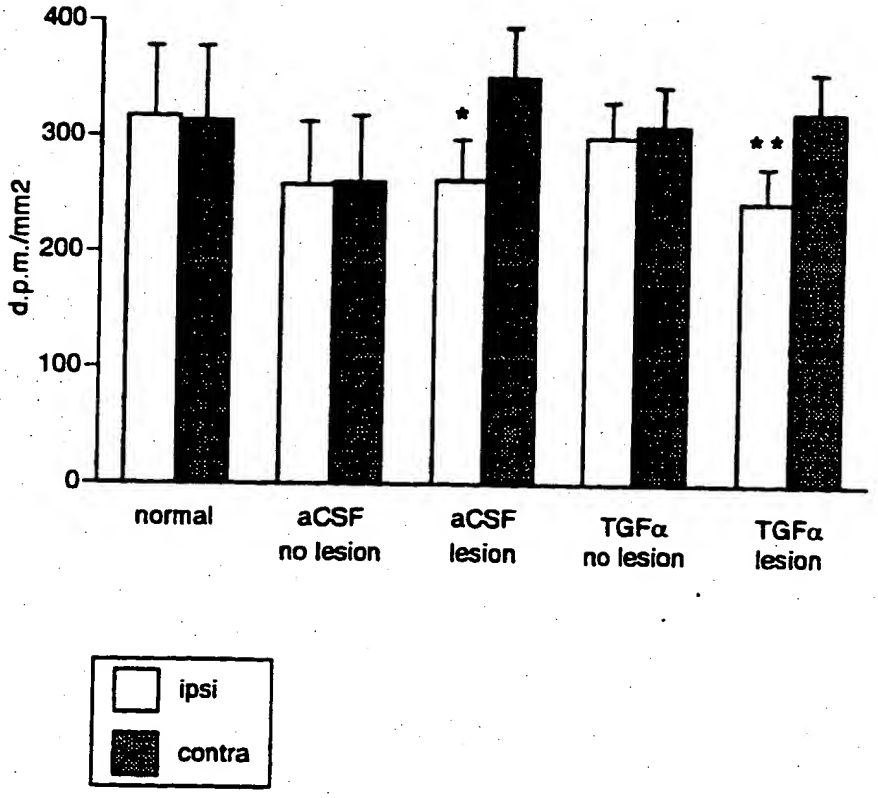


Figure 3.17. Bar graph showing the average standardized densities of EGF receptor mRNA hybridization in the non-ridge bodies of the striata in each of the five groups examined. White bars represent averages from the ipsilateral striata. Filled bars denote averages from contralateral striata. Average hybridization density was unchanged from normal in any of the experimental groups. Averages ± S.E.M. (Student's *t*-test, paired for ipsilateral-contralateral comparisons).

EGF Receptor mRNA Hybridization Densities
in the Non-Ridge Striatum

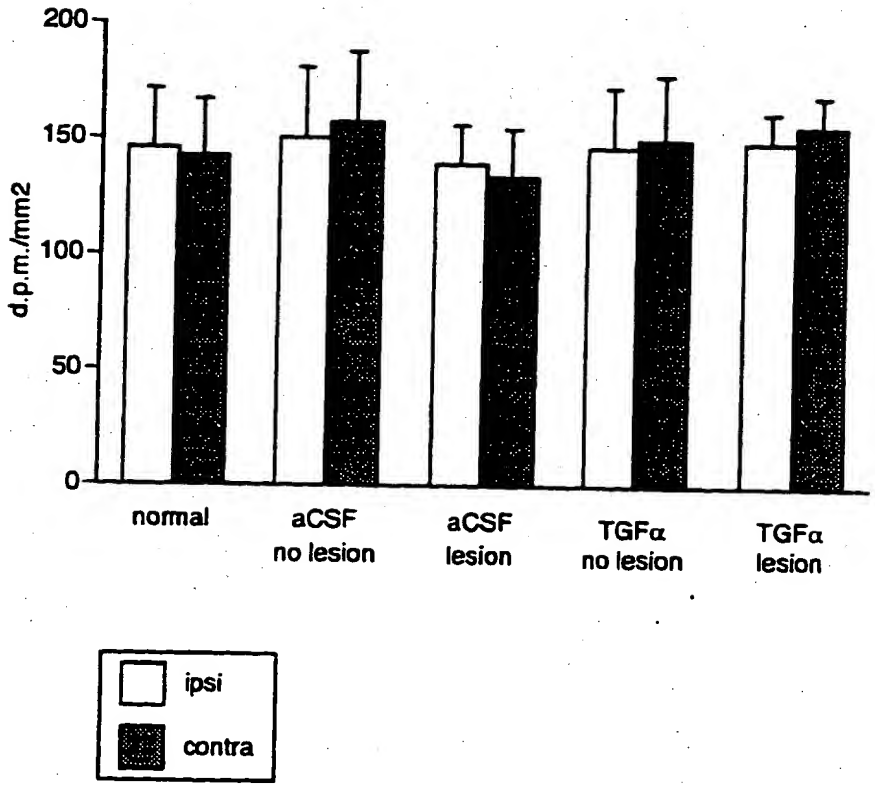


Figure 3.18. Bar graph showing the average standardized densities of EGF receptor mRNA hybridization in the subependymal regions along the edges of the striata bordering the lateral ventricles. White bars represent averages from the ipsilateral striata. Filled bars denote averages from contralateral striata. Average hybridization density was approximately doubled in the ipsilateral subependymal region in both groups receiving TGFα striatal infusions. Averages ± S.E.M. (Student's *t*-test, paired for ipsilateral-contralateral comparisons; P values, * $p < 0.01$, ** $p < 0.0001$).

EGF Receptor mRNA Hybridization Densities
in the Subependymal Zone

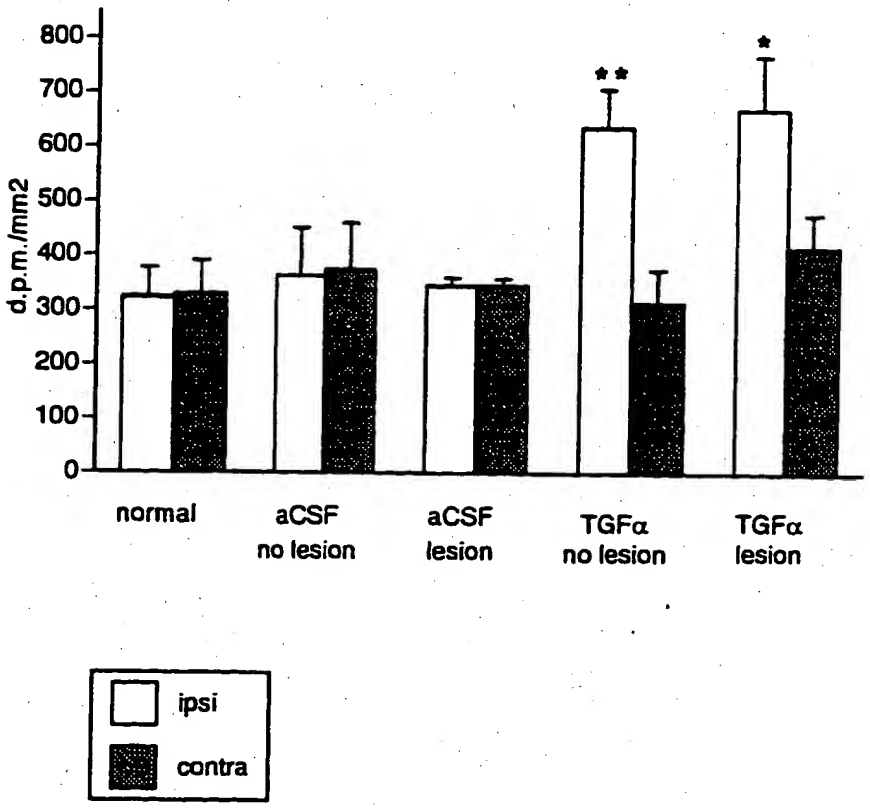
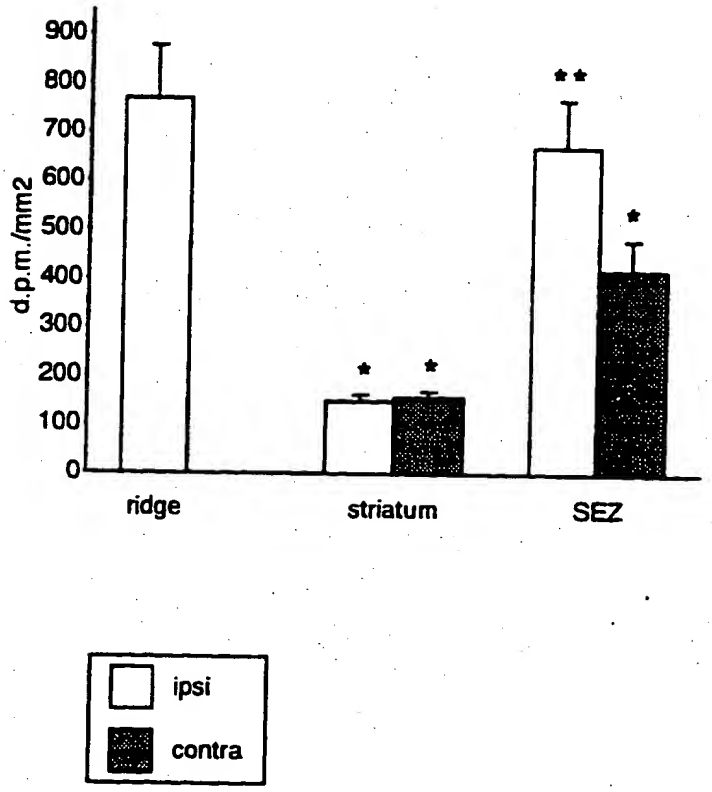


Figure 3.19. Bar graph comparing the average standardized densities of EGF receptor mRNA hybridization in the striatal ridges, the non-ridge body of the striatum and the subependymal regions in all animals with striatal ridges. White bars represent averages from the ipsilateral striata. Filled bars denote averages from contralateral striata. Average hybridization density was highest in the striatal ridge ipsilateral to the treatments. No striatal ridges ever appeared in the contralateral striata. TGFα striatal infusions. Averages ± S.E.M. (Student's *t*-test, paired for ipsilateral-contralateral comparisons; P values, * $p < 0.005$, ** $p < 0.001$).

EGF Receptor Hybridization Densities in the Striatal Ridge, the Non-Ridge Striatum, and the Subependymal Zone in All Animals with Striatal Ridges



Discussion

We have studied the modulatory effects of nigral 6-OHDA lesions or striatal infusions of TGFα, or both, on the expression of mRNAs encoding TGFα and the EGF receptor in the adult rodent nigrostriatal system. The results clearly demonstrated changes in expression associated with each treatment individually, and a unique pattern of striatal expression when the two treatments were combined.

Effects of 6-OHDA lesions. Midbrain lesions with 6-OHDA reduced TGFα mRNA hybridization in the striatum by 25 percent at several weeks post-lesion. If TGFα peptide levels parallel expression of TGFα mRNA in this system, the decrease in TGFα mRNA may be one aspect of the rodent lesion model that is not similar to idiopathic human PD: TGFα is greatly *increased* in the striata of PD patients (Mogi et al., 1994). TGFα has been shown to enhance a number of measures of dopamine neuron function *in vitro* (Alexi and Hefti, 1993). The increase of TGFα (and EGF and other trophic factors) may therefore reflect a response to the continuing degeneration of dopamine neurons and their striatal efferents and may contribute to the capacity of remaining midbrain dopamine cells to compensate for the lost striatal dopaminergic innervation (Mogi et al., 1994). Thus, a partial- or perhaps a chronic-injury model might better represent this aspect of human PD.

The difference in the time course of the loss of dopamine cells may also help explain the apparent discrepancy between the 6-OHDA rodent model and

human PD. In the rat model, midbrain dopamine neurons are killed relatively quickly by a single injection of neurotoxin. The chronic, progressive degeneration of mesencephalic dopamine neurons in human PD occurs over many years. In the present study, the animals were sacrificed well after midbrain dopamine cells had degenerated. There may have been early changes in striatal TGFα mRNA expression in these animals that would not be apparent in our experiments. It would be of interest to determine how TGFα mRNA expression varies in the rat model over shorter periods post-lesion, while dopamine neurons are in the process of degenerating.

Moderate TGFα mRNA expression in the caudate-putamen is consistent with its putative role as a target-derived growth factor for midbrain dopamine neurons. The underlying cause for its decrease in the ipsilateral striatum after midbrain neurotoxic lesion is unclear. Dopamine receptor binding has been shown to influence TGFα mRNA expression in the hypothalamus (Borgundvaag et al., 1992), but no such interaction has yet been demonstrated in the striatum. TGFα mRNA is expressed in subpopulations of neurons and glia in the normal adult rodent striatum (Seroogy et al., 1993). Dopamine denervation of the striatum could potentially have influenced TGFα mRNA expression in postsynaptic neurons, astrocytes or both.

Contralateral striatal TGFα mRNA expression was not significantly altered by 6-OHDA lesion. This finding, too, is consistent with a dopamine denervation-mediated decrease in TGFα mRNA expression. Only a few

percent of mesostriatal dopaminergic projections are contralateral (Loughlin and Fallon, 1982), thus any contralateral regulatory effects resulting from the lesion would be expected to be minor compared to ipsilateral effects.

EGF receptor mRNA hybridization in the DA-denervated striatum did not differ significantly from that in the contralateral CP or in unlesioned control striata. Again, there may have been early changes in expression due to the midbrain lesion or implantation of the infusion cannula that were not manifest at several weeks postlesion or two weeks post-implantation. The abolition of EGF receptor mRNA hybridization in the lesioned SNc confirms a similar observation after 6-OHDA lesion of the medial forebrain bundle (Seroogy et al., 1994). This lesion-induced decrease was previously cited as evidence of EGF receptor expression by nigral dopamine neurons (Seroogy et al., 1994), but the possibility remains of nigral glial production of EGF receptor mRNA that is subject to regulation by injury or death of nearby nigral dopamine neurons. Interestingly, EGF receptor binding in the midbrains of postmortem PD patients is unchanged from normals (Villares et al., 1993). Thus, the loss of EGF receptor mRNA expression after lesion represents another difference between the rodent lesion model and human PD. As with TGFa expression in the striatum, a partial-lesion model may better mimic in a rat brain the changes seen in a Parkinsonian human brain.

Effects of TGFα infusions. In unlesioned animals, infusion of TGFα or aCSF did not significantly alter TGFα mRNA hybridization from normal levels in the midbrain or striatum. Despite reports of autostimulation of TGFα expression in

other tissues or cells types (Coffey et al., 1987; Bjorge et al., 1989; Untawale et al., 1993; Dlugosz et al., 1994; Barnard et al., 1994), results from the present study do not provide evidence for such activity in this system. The autostimulatory effects in those earlier studies were produced on the order of a few hours. Brain tissue in the present study was obtained after continuous exposure to the growth factor over a period of two weeks. Thus, an early upregulation near the beginning of the infusion that later subsided would not be evident.

As with TGFα and EGF receptor transcripts in lesion-only animals, it would be of interest to examine the time course of modulation at time points earlier after onset of the experimental treatment. The slight increase in TGFα mRNA seen in a few animals immediately around the infusion scar was found in both TGFα- and aCSF-infused striata. Thus it is probably attributable to continued mechanical injury and gliosis caused by the cannula itself and not to the infusate.

TGFα infusions dramatically increased EGF receptor mRNA hybridization in the ipsilateral subependymal zone but not in the rest of the striatum. In other tissues, EGF receptor mRNA can be modulated by several chemical and mechanical means. EGF peptide increased EGF receptor mRNA in numerous mammalian cell types *in vitro* (Earp et al., 1986; Fernandez-Pol et al., 1987; Bjorge et al., 1989; Thompson and Rosner, 1989; Kesavan et al., 1990). Retinoic acid caused a similar increase in normal rodent fibroblasts (Thompson and Rosner, 1989) and in a transformed rat liver cell line (Raymond et al.,

1990). Exposure to cycloheximide, by itself or with EGF, stimulated an increase in cultured human cytotrophoblasts and stabilized EGF receptor transcripts, thus providing a mechanism other than enhanced transcription to increase total abundance of EGF receptor mRNA (Kesavan et al., 1990).

Transection of the sciatic nerve in rats brought about a graded increase in EGF receptor mRNA in the severed ends (Toma et al., 1992). Treatment with protamine increased ^{125}I-EGF binding and cell surface receptor number in mouse and human cell lines *in vitro* (Lokeshwar et al., 1989). TGFα may mimic these actions and increase the production and/or longevity of EGF receptor transcripts in extant subependymal cells. It may also stimulate transcription in cells that do not normally express appreciable amounts of EGF receptor mRNA. Both of these effects could be investigated further *in vitro*.

An additional possibility is that TGFα is stimulating proliferation of subependymal cells and increasing the total numbers of cells expressing EGF receptor mRNA. EGF and TGFα are potent mitogens for cultured "EGF responsive" cells explanted from the subependymal region (Reynolds and Weiss, 1992; Reynolds et al., 1992). The strong inductive effect striatal TGFα infusion had on subependymal EGF receptor mRNA hybridization may indicate that proliferative cells in the intact brain respond similarly to these cells *in vivo*.

Combined TGFα infusion and 6-OHDA lesion. In animals receiving combined lesions and TGFα infusions, striatal TGFα mRNA hybridization was indistinguishable from that in animals receiving combined lesions and aCSF

infusions. Although TGFα has potent autostimulatory effects in other tissues, it did not significantly alter the reduction of striatal TGFα mRNA hybridization in the present study. The 6-OHDA-mediated loss of mesencephalic EGF receptor mRNA was similarly unaffected by TGFα infusion. In the latter case, the midbrain lesions were performed, and ipsilateral dopamine cells destroyed, weeks prior to the start of the infusion. Thus, the growth factor would not have had an opportunity to prevent their elimination.

There is some evidence that the dopamine cells themselves express EGF receptor mRNA (Seroogy et al., 1994) and that TGFα can moderate the loss of markers for striatal dopaminergic innervation if administered concurrently with the neurotoxin (Reid et al., 1997). Therefore, the time interval between the neurotoxic lesions and the administration of TGFα may explain why the TGFα infusions had no impact on the abolition of midbrain EGF receptor mRNA.

In human Parkinsonian brains, mesencephalic EGF receptor binding is unchanged from normals (Villares et al., 1993). The huge increases in striatal TGFα (and other neurotrophic factors) with PD (Mogi et al., 1994) may mask a reduction in the number of EGF receptor expressing dopamine cells by increasing the levels of expression in the remaining neurons. On the other hand, *in vitro* experiments suggest that many of the trophic effects of TGFα on mesencephalic dopamine neurons are mediated, at least partially, through glia (Alexi and Hefti, 1993). TGFα may therefore act through paracrine (direct) and sequential (indirect) modes of transport to influence dopamine neurons.

The pattern of EGF receptor mRNA hybridization in the subependymal zone of TGFα/lesion animals was similar to that seen in TGFα/nonlesion animals. The most striking feature in the ipsilateral striata of these animals was a dense ridge of hybridization well out in the body of the striatum, more intense even than the enhanced hybridization in the subependymal zone. The ridge did not correspond to any known anatomical feature and was not evident with the TGFα or TH probes. EGF receptor mRNA hybridization in the non-ridge striatum was the same as in the striata of all other groups.

The neurotoxic damage from the 6-OHDA lesions and the mechanical injury from implantation of the infusion cannula may have stimulated proliferation and activation of glial cells. Previous studies have demonstrated gliosis and increased astrocytic EGF receptor expression as a result of injury (Nieto-Sampedro et al., 1988; Fernaud-Espinoza et al., 1993). Further, TGFα may play a role in the reactivity of astrocytes (Junier et al., 1994). The shape of the ridge may be related to the infusion cannula scar.

Another possibility is that proliferative cells of the subependymal region were drawn away from the ventricle and into the overlying striatum by the combined growth factor infusion and midbrain lesion. TGFα is a potent chemoattractant for diverse cell types (Grotendorst et al., 1989; Reneker et al., 1995), but by itself was not sufficient in most animals to stimulate formation of the ridge. Formation of the cellular ridge may have been facilitated by the midbrain lesions. The origin and identity of these cells will be examined in the following chapter.

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CHAPTER FOUR

CHARACTERIZATION OF THE STRIATAL RIDGE

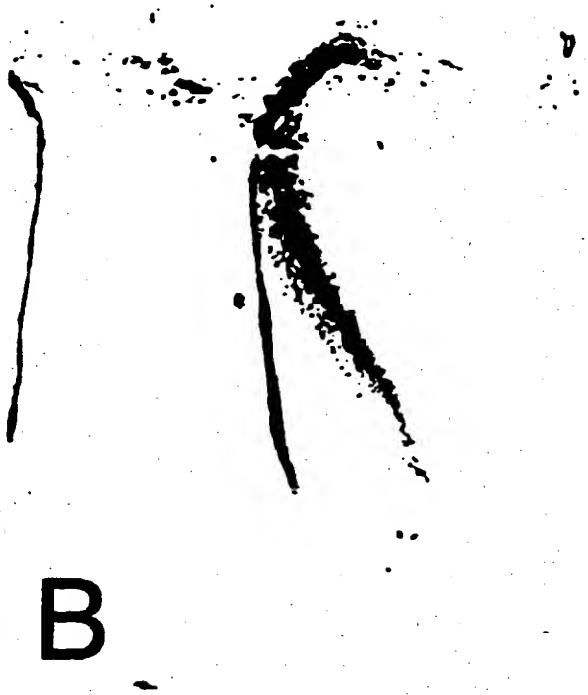
In the previous chapter, striatal infusions of TGFα, when combined with nigral 6-OHDA lesions, induced the formation of a dense ridge of cells in the body of the striatum that abundantly expressed EGF receptor mRNA (Figure 4.1) but no more TGFα mRNA than the surrounding tissue. The ridge was comprised of a mass of densely packed cells, allowing its clear detection using simple thionin staining (Figure 4.1). The identity of the anomolous striatal ridge was not apparent, but three possibilities were considered.

Gliosis in response to injury is a feature of both traumatic and neurotoxic damage to brain tissue. Typically, both types of brain injury stimulate astrocytosis and infiltration of injured tissue by astrocytes and microglia (Fernaud-Espinoza et al., 1993). Astrocytes have been shown to express EGF receptor immunoreactivity, particularly in response to brain injury (Gómez-Pinilla et al., 1988; Nieto-Sampedro et al., 1988). In addition, TGFα itself stimulates the proliferation of astrocytes (Alexi and Hefti, 1993). Therefore, the possibility that the striatal ridge was a mass of glial cells responding to the combined neurotoxic and mechanical damage and infusion of the growth factor was considered.

A second potential source for the ridge was investigated that was related to the distinctive anatomy of the rodent striatum. The ridge did not correspond to

Figure 4.1. Photomicrograph of an autoradiogram of EGF receptor mRNA *in situ* hybridization and thionin staining in the same coronal section through the adult rat forebrain. (A) The striatal ridge can be plainly seen due to its intense hybridization. (B) Thionin staining also reveals the ridge.


A


B

any previously identified anatomical feature. In rodents, the caudate and putamen are not anatomically distinct structures. No anatomical or neurochemical markers have been identified thus far that distinguish between these two nuclei of the basal ganglia in rodents. However, during prenatal and early postnatal development, neurogenetic gradients within different regions of the developing striatum correspond to characteristic gradients in the caudate and putamen in animals where these nuclei are anatomically discrete (Bayer, 1984). In rodents, new striatal neurons rostral to the decussation of the anterior commissure are added in a lateral-to-medial gradient such that the latest born neurons are those nearest the lateral ventricle. That same pattern is observed in the development of the caudate nucleus, suggesting that the anterior striatum in rodents is more of a "caudate-like" region. Caudal to the crossing of the anterior commissure, neurons are added in a medial-to-lateral gradient, similar to the developing putamen in animals where it is anatomically distinct. Thus, the posterior rodent striatum may be more "putamen-like". The possibility was considered that the striatal ridge, then, might represent a previously unrecognized border between these two regions of rat striatum that allowed a dense buildup of cells, perhaps due to some neurochemical difference.

A third possibility for the source of the striatal ridge was also examined. Explanted cells from the subependymal zones of the forebrain lateral ventricles of adult mammals have been found capable of proliferating and differentiating into new neurons and glia, particularly when cultured in the presence of EGF-family neurotrophic factors, including TGFα (Reynolds et al., 1992; Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994).

Recently, EGF or TGFα infused into the lateral ventricle or striatum stimulated proliferation of "EGF-responsive" stem and neural progenitor cells in the adult mouse brain (Craig et al., 1996). An exciting possibility for the source of the striatal ridges in our studies was that the TGFα infusions stimulated similar proliferative activity in the brains of rats. Additionally, we considered whether the striatal ridges were mass migrations of proliferating neural progenitor cells derived from the subependymal regions.

The experiments presented in this chapter sought to characterize this anomolous striatal ridge using a variety of histochemical and immunohistochemical techniques. The origin of the ridge and the factors influencing its appearance were also investigated by examining the time course of its formation in the striatum and by altering the combinations of surgical and chemical treatments.

Materials and Methods

The animals and experimental procedures in this study were approved by the University of California, Irvine, Animal Research Committee in accordance with National Institutes of Health guidelines. Adult male Sprague-Dawley rats (250-350 g) were used throughout the study. Twenty-four animals received standard midstriatal infusions of rat TGFα (0.5 $\mu g\ d^{-1}$) (Sigma, Inc.). Another twenty-six rats received either artificial cerebrospinal fluid (aCSF) or no infusion. A subset of animals in the standard TGFα infusion group and the control group received stereotaxic 6-OHDA injections into the substantia nigra

48 hours after the infusions were begun. Animals used in this portion of the study were classified into six groups according to their infusion/lesion combination as follows: TGFα infusion, lesion (n=13) TGFα infusion, no lesion (n=11); aCSF infusion, lesion (n=12); aCSF infusion, no lesion (n=9); no infusion, lesion (n=1); no infusion, no lesion (n=4).

Additional animals received TGFα infusions into other regions of striatum, the lateral ventricle, cerebral cortex, or the septum. Four more animals (two per group) received midstriatal TGFα infusions at one-half or one-tenth the standard dose. Also, two animals received midstriatal infusions of epidermal growth factor (EGF) instead of TGFα. The EGF administered in these rats was at the standard 0.5 μg d^{-1} dose. All of the animals in these extra groups were lesioned. Rats in all of the experiments were typically perfused one to sixteen days postlesion (three to eighteen days of infusion). To determine whether the ridge would persist after the infusions ceased, four animals with TGFα infusions and 6-OHDA lesions had their minipumps removed at the end of two weeks, but were not perfused until several weeks later. Microtomed brain sections were prepared and stained using various immunocytochemical and histochemical techniques.

TGFα infusion. Rats were anesthetized with 8 mg xylazine and 100 mg ketamine per kilogram body weight. Infusions of TGFα were provided up to 18 days by Alzet osmotic minipump (2002). The minipumps were filled to about 200 μl with either aCSF for control animals or 20 μg TGFα in 400 μl of aCSF

(50 μg/ml) for experimental animals. Under sterile conditions, the infusion cannula was positioned to stereotaxic coordinates (+1.2 A/P; +2.7 M/L; –6.0 D/V) based on Bregma (Paxinos and Watson, 1986) and cemented to the top of the skull with dental cement. The infusate was delivered via cannula at approximately 0.5 μl h^{-1}. Some additional control animals received infusions either into the lateral ventricle, the overlying cortex or other areas.

Neurotoxic lesion. Forty-eight hours after the minipump implant, rats were anesthetized as above. A chilled 4.8 mg/ml solution of 6-OHDA HCl was prepared immediately prior to injection. Using sterile technique, the neurotoxin was stereotaxically injected into the ipsilateral substantia nigra (SN) (+3.7 A/P; +2.1 M/L; +2.0 D/V) using interaural zero as a reference (Paxinos and Watson, 1986). A 6-8 μl volume was injected at a rate of 1 μl min^{-1}.

Tissue preparation. Animals were perfused with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.4) one to sixteen days postlesion and their brains placed into 20% sucrose. The next day, the brains were frozen in isopentane at -20°C. Forty-micron coronal sections were then cut on a freezing microtome into 2% paraformaldehyde in 0.1 M PBS. Continuous sections were taken through the striatum and substantia nigra-ventral tegmental area (SN-VTA). Representative sections were taken through the rest of the brain.

Nissl staining. Microtomed brain sections for Nissl staining were mounted onto gelatin-coated slides and allowed to dry overnight. They were then dehydrated and rehydrated through ethanol baths, and placed in Thionin solution for approximately four minutes. The sections were dehydrated through the series

of ethanol baths, cleared in successive Histoclear washes, and coverslipped. Sections were viewed under light microscopy and photographed with Technical Pan Film (Kodak, Inc.) at ISO 100 (HC-110 processing for six minutes).

Silver staining. Degenerating fibers and cells of the ridge were labeled using a modification to the Nauta stain method, similar to Procedure I of Fink-Heimer (Giolli and Karamanlidis, 1978). Briefly, free-floating sections were placed into 0.05% potassium permanganate prior to treatment with fresh 1% hydroquinone/1% oxalic acid. They were treated with successive uranyl nitrate/silver nitrate solutions of increasing concentration. After another rinse, the sections were reacted in ammoniacal silver, then in ethanol/citric acid/paraformaldehyde reducer, and finally in sodium thiosulfate. After staining, they were mounted on glass slides and allowed to dry on a slide dryer for 15 min. The sections were then dehydrated through successive ethanol washes of increasing concentration, defatted in three successive Histoclear washes, and coverslipped.

Immunohistochemistry. The quality and the extent of the nigral lesion were determined by the loss of tyrosine hydroxylase immunoreactivity (TH-IR) in the ipsilateral ventral midbrain. Antibodies against glial fibrillary acidic protein (GFAP), a marker for astrocytes, nestin, a marker for neural progenitor cells, and vimentin, a marker for radial glial cells, were employed in the neurochemical characterization of the ridge. Immunohistochemistry was performed on free-floating sections. Briefly, brain sections were washed in 0.1M PBS or Tris buffered saline (TBS) (3x10 min.) then incubated for 1 h in blocking solution consisting of 3% normal goat serum in 0.1M PBS or TBS

with 250 µl Triton X-100. Next, they were incubated overnight at room temperature on a rotator with antibody solution diluted with blocking solution: rabbit anti-TH antiserum (Eugene Tech Intl., Inc.) (1:500), rabbit anti-GFAP (Dako Corp.) (1:6400), mouse monoclonal anti-vimentin (Sigma Chemical, Inc.) (1:50) or with mouse anti-nestin supernatant (U. Iowa, Hybridoma Bank) (1:20).

The sections then were washed and incubated for 1 h with biotinylated goat anti-rabbit antiserum (Vector Labs, Inc.) (1:200) for TH or GFAP immunostaining, or biotinylated horse anti-mouse antiserum (Vector Labs, Inc.) for nestin or vimentin immunostaining, then washed and incubated in avidin-biotin complex (ABC Elite kit, Vector Labs, Inc.) for 1 h. Localization of primary antibody binding was revealed using the diaminobenzidine (DAB) peroxidase technique. The sections were washed thoroughly and mounted on gelatin-subbed slides and allowed to dry overnight. Finally, the sections were dehydrated, cleared and coverslipped as described above.

Results

None of the animals used in the study displayed adverse effects from the minipump implants or lesion surgeries. All continued to take food and water through the course of the experiments. If the lesion, infusion or both were not successful the animals (n=6) were excluded from the initial experimental groups and examined separately. A successful lesion was defined as one that caused complete or near-complete elimination of ipsilateral nigral TH-IR. A

successful striatal infusion was defined as one where the tip of infusion cannula was successfully fixed into the body of the striatum.

Striatal infusions. As with the previous *in situ* hybridization studies, all animals receiving intrastriatal TGFα infusions of six days or more displayed a dramatic buildup of cells along the ventricle ipsilateral to the infusion, visible with thionin staining (Figure 4.2). By comparison, the contralateral striatum showed no such increase (Figure 4.2), and was indistinguishable from that in aCSF-infused animals. EGF infusions in lesioned animals induced the cellular expansion along the ventricle, but did not induce formation of the striatal ridge. Lower doses of TGFα induced both the cellular expansion and ridge formation, but, qualitatively, the number of cells in each was decreased.

Effects of 6-OHDA lesions. None of the lesioned animals receiving aCSF infusions showed any cellular expansion along the ipsilateral ventricle or any evidence of a striatal ridge. Lesioned animals infused with TGFα did uniformly exhibit the buildup of cells along the ventricle and typically displayed the striatal ridge. Nigral lesions dramatically increased the incidence of formation of the ridge compared to unlesioned animals (Table 4.1).

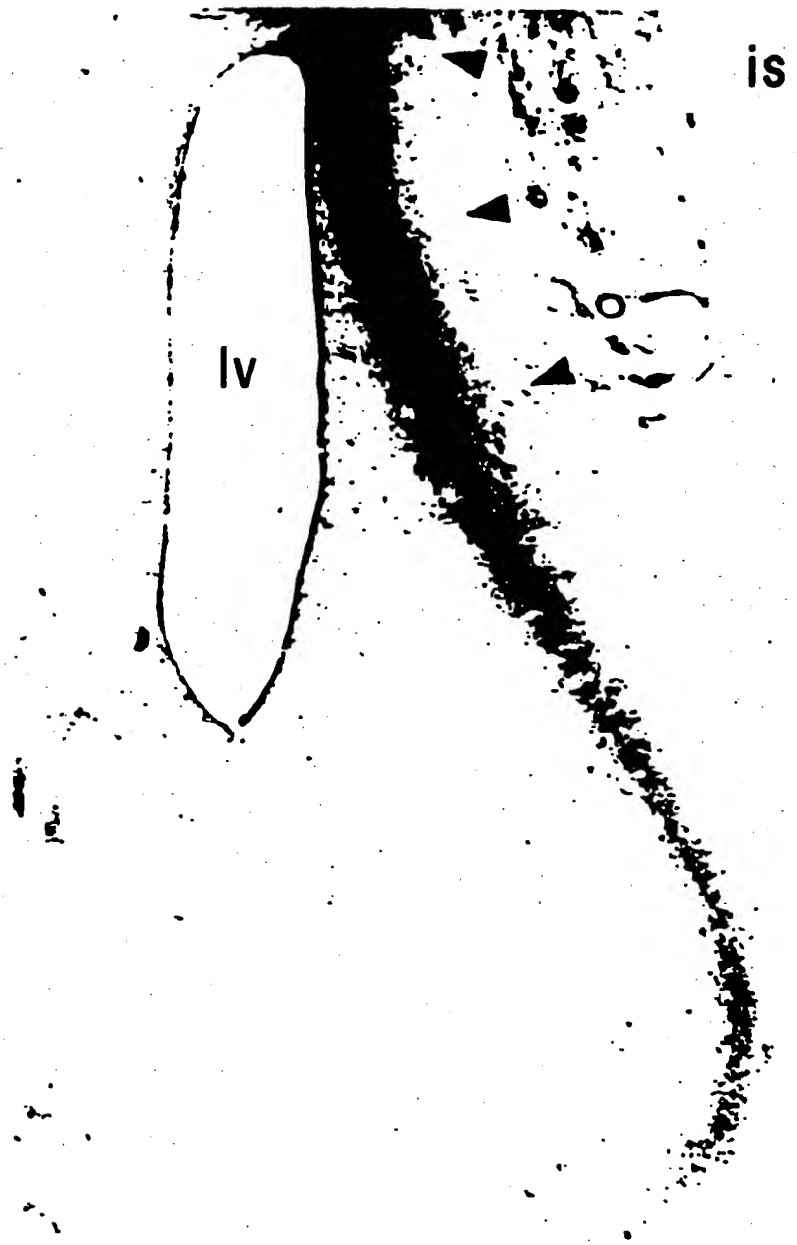
Morphology and persistence of the ridge. Midstriatal infusion resulted in a characteristic S-shaped ridge arising from the dorsomedial caudate-putamen, sweeping out into the striatum and looping back slightly toward the midline at its ventral end (Figure 4.3). The dorsal-most portion of the ridge was continuous with the build-up of cells in the subependymal region. Typically, thionin staining was most dense in the dorsal portion of the ridge (Figure 4.3)

Figure 4.2. Photomicrograph of a thionin stained coronal section through the striatum of an adult rat receiving nigral 6-OHDA lesion and striatal infusion of TGFα for six days. A dense expansion of Nissl staining is seen along the ipsilateral subependymal region. The expansion is most dense along the dorsomedial border of the striatum (arrowheads). The infusion scar (is) is seen lateral to the cellular expansion. No evidence of a striatal ridge was seen in any of the animals infused for six days or less.

Table 4.1. Summary of the percentages of animals in each group exhibiting the dense cellular expansion along the subependymal zone or the striatal ridge in the body of the striatum.

Infusate	Lesion	Number	Expansion	Ridge
aCSF	no	9	0%	0%
aCSF	yes	12	0%	0%
TGFα	no	11	100%	27%
TGFα	yes	13	100%	92%

Figure 4.3. Photomicrograph of thionin staining in a coronal section though the adult rat striatum in an animal receiving a nigral 6-OHDA lesion and a fourteen-day continuous infusion of TGFα. In the striatal ridge as in the subependymal cellular expansion in Figure 4.2, the densest staining was seen in the dorsal portion (arrowheads). (lv, lateral ventricle; is, infusion scar).


is
o
lv

paralleling the EGF receptor mRNA hybridization. The cellular ridge was generally found throughout most of the rostral-caudal extent of the striatum. The ridge was still prominent in the striatum three months after the TGFα infusion pump was removed.

GFAP immunohistochemistry. Antiserum against glial fibrillary acidic peptide (GFAP), a marker for astrocytes, failed to stain cells of the striatal ridge or the cellular expansion along the ventricle. Normal GFAP-IR astrocytic staining was found medial and lateral to the ridge, but was nearly excluded from the ridge itself (Figure 4.4).

Silver staining. Labeling cells non-specifically with a modification of the Nauta method provided additional information about the cells comprising the ventricular cellular expansion and the striatal ridge. One of the most striking features was the huge number of cells making up the subependymal cellular buildup and ridge (Figure 4.5). The cells were densely packed and predominantly fusiform in shape (Figure 4.5). In the ventral portion of the ridge, elongated cells appeared to stream around fiber bundles of the internal capsule suggesting that the cells were migrating through the striatum (Figure 4.6).

Time course of ridge formation. The density of cells of the ridge allowed us to track its formation using simple thionin staining (Figure 4.7). All animals used for the time-course experiment received 6-OHDA lesions and midstriatal TGFα infusions. At time points prior to six days of infusion, there was only a very minor build-up of cells in the subependymal region and no evidence of a striatal ridge. By six days of infusion, there was a clear expansion of cells along the

Figure 4.4. Photomicrograph of glial fibrillary acidic peptide (GFAP) immunostaining in a coronal section of adult rat brain infused for fourteen days with TGFα and lesioned with 6-OHDA in the substantia nigra. Normal astrocytic staining is seen medial to the striatal ridge near the lateral ventricle (lv), and lateral to the ridge. GFAP-IR is markedly reduced in the region of the ridge (arrowheads).

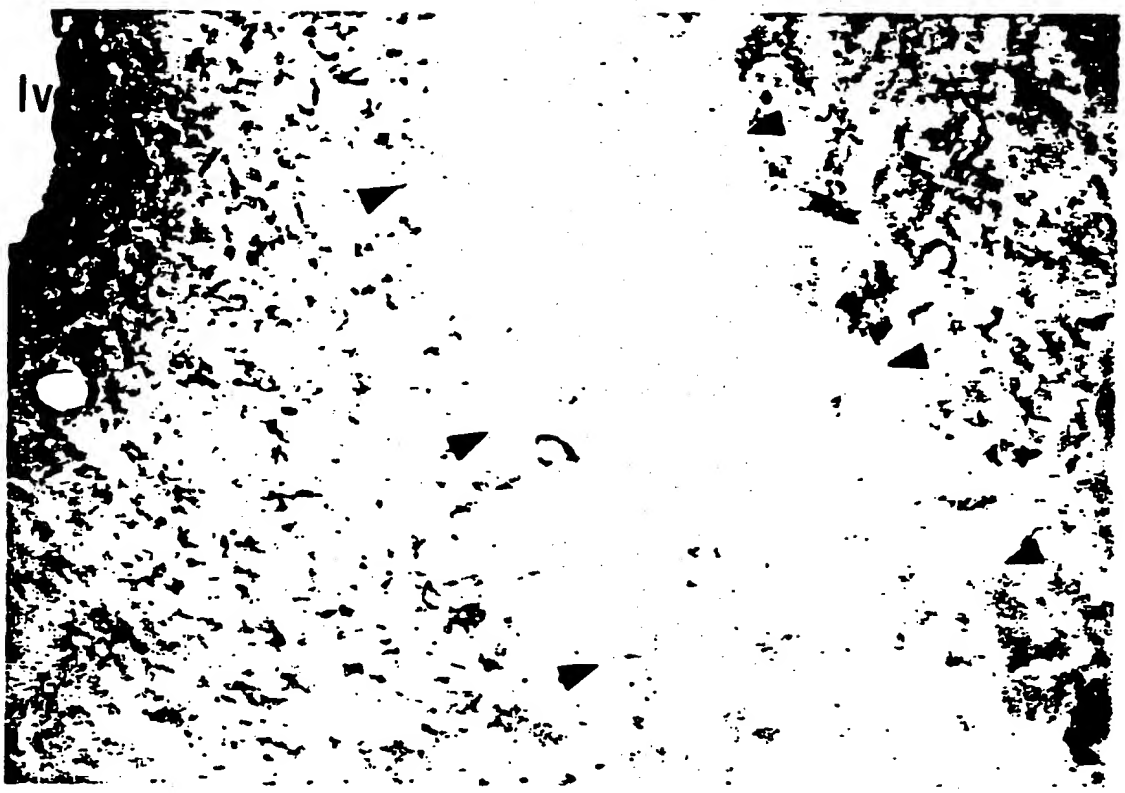

lv

Figure 4.5. Photomicrograph of silver staining in a coronal section through the striatum in an adult rat receiving nigral 6-OHDA lesion and fourteen days of TGFα infusion. In the caudate-putamen ipsilateral to the treatments (A), huge numbers of stained cells are seen comprising the dorsal portion of the ridge. Many of the cells of the ridge exhibit elongated morphologies and are oriented normal to the subependymal region. There is also an increase in the number of cells along the lateral ventricle (lv). In the contralateral striatum (B), no expansion of cells is seen either in the striatum or along the lateral ventricle.

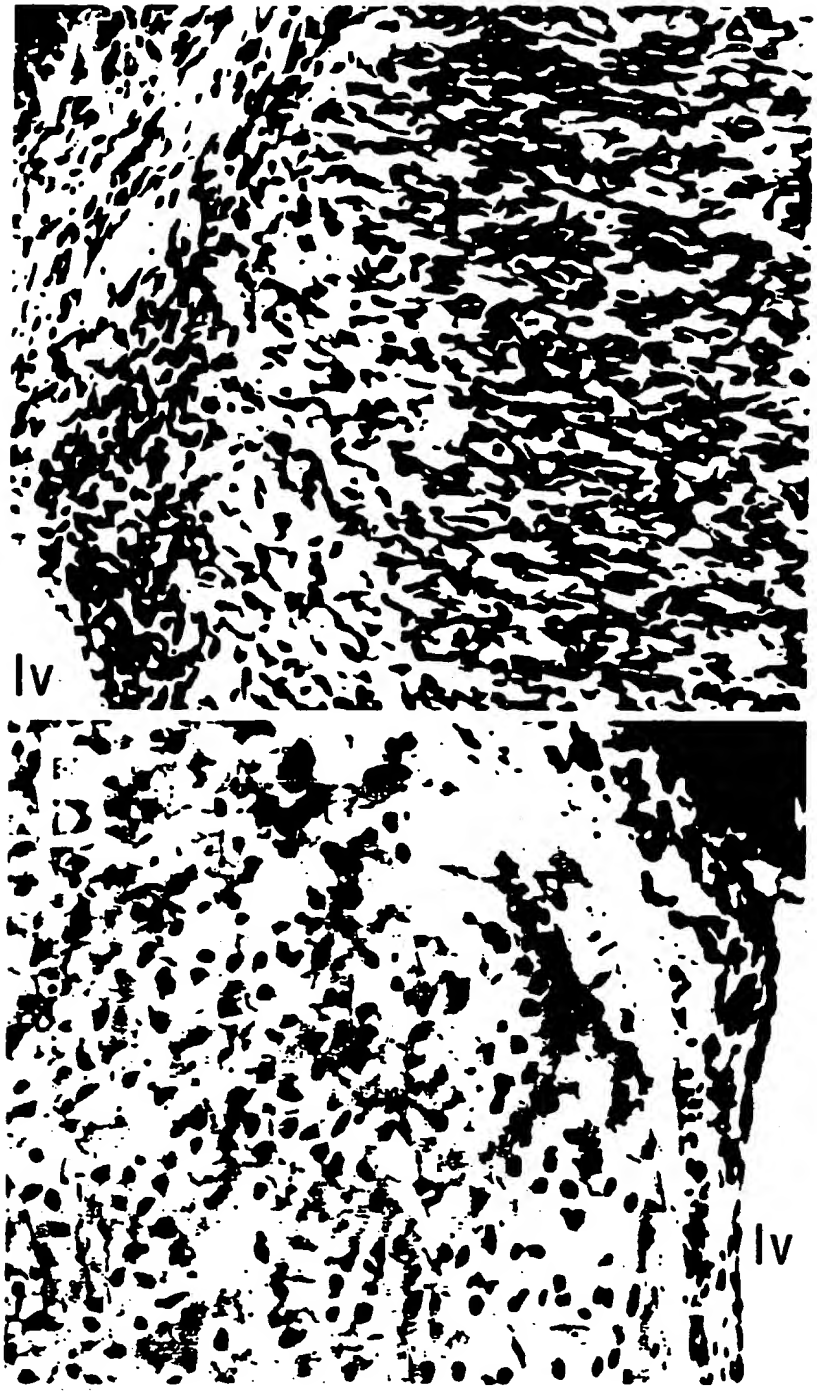

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Figure 4.6. Photomicrograph of silver staining in a coronal section through the adult rat striatum in an animal receiving striatal infusion of TGFα for fourteen days and nigral lesion with 6-OHDA. The field shown is of a ventral portion of the ridge. The number and density of fusiform cells in this region is much reduced compared to more dorsal segments of the ridge. Elongated cells appear to stream around dense fiber bundles of the internal capsule.

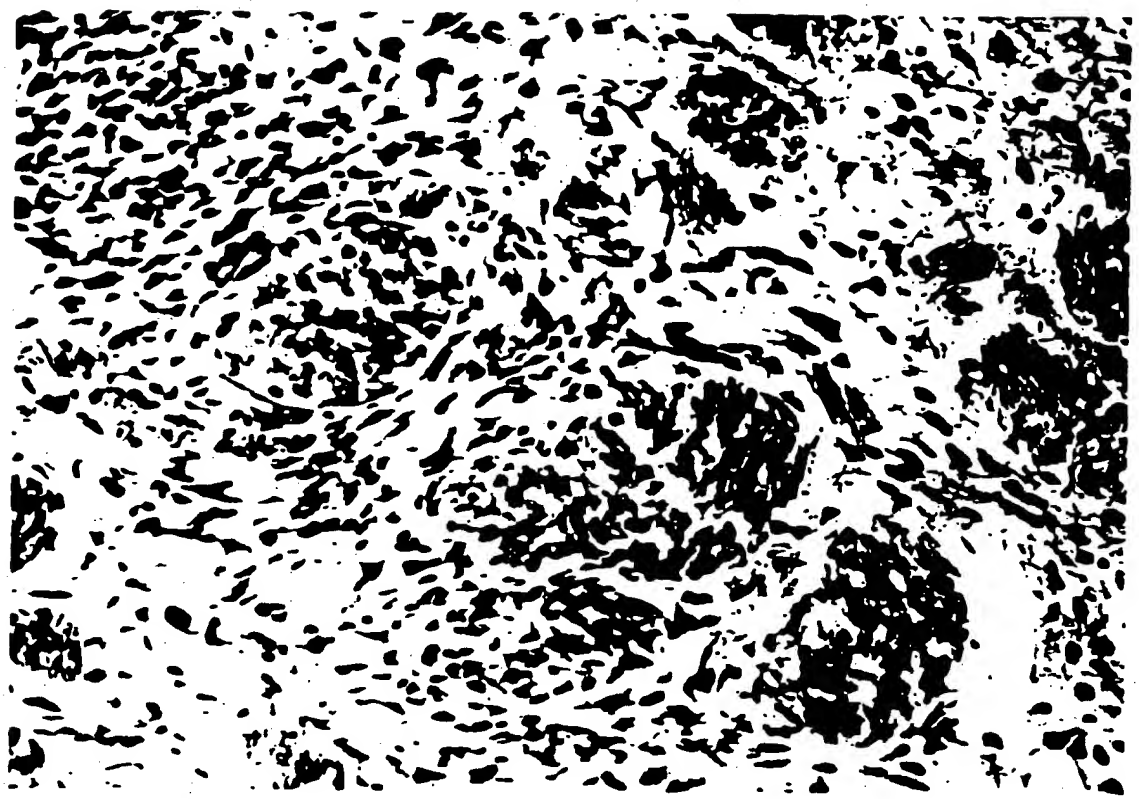
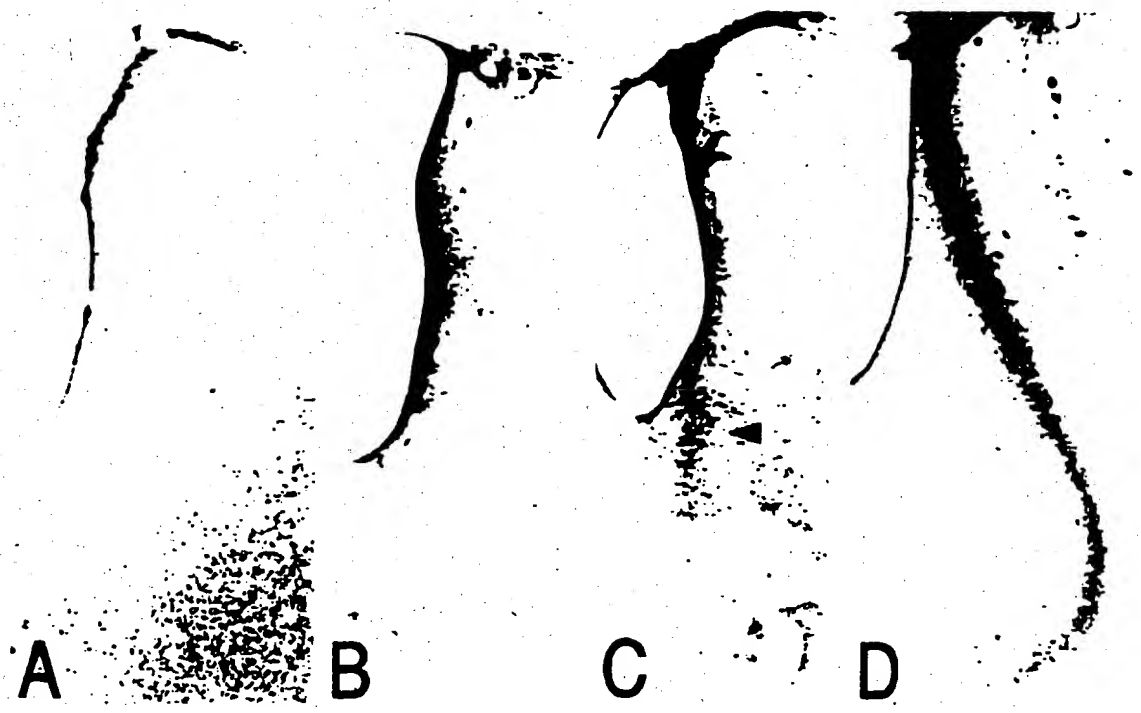


Figure 4.7. Photomicrographs of thionin-stained coronal sections of adult rat brain in animals receiving nigral 6-OHDA lesions and striatal infusions of TGFα for various duration. After four days of infusion (A), the cellular expansion in the subependymal region was barely detectable above background staining. At six days of infusion, the build-up of thionin-stained cells near the lateral ventricle was much more robust and was easily detected. By the ninth day of infusion (C), a region of densely-stained cells appeared slightly lateral to the subependymal zone at the ventral end of the cellular expansion. At fourteen days of infusion (D), a dense, well-formed ridge was evident well into the body of the striatum.


A
B
C
D

ventricle. At nine days infusion, the ventral portion of the ridge had begun to appear slightly displaced from the ventricle. By twelve days infusion, the ventral portion of the ridge was situated as much as 400 μm from the ventricle wall. At sixteen days infusion, the ridge appeared midstriatum, its ventral portion up to two millimeters from the ventricle. Thus the ridge originated in the ventricular region and was increasingly displaced radially in the overlying striatum at greater times of infusion. The estimated difference in distance between the lateral extents of the ridges and the ventricle wall at twelve days and sixteen days of infusion was approximately 1.6 mm.

Nestin immunohistochemistry. Monoclonal antibodies against nestin, a marker for neuroepithelial progenitor cells, intensely stained dense collections of fibers throughout the ridge (Figure 4.8). No nestin-IR fibers were seen lateral to the ridge, but occasional fibers were observed medial to the ridge (Figure 4.9). The fibers were oriented primarily orthogonal to the ridge (Figure 4.9).

Alteration of ridge morphology. Lesioned animals infused midstriatally with TGFα uniformly exhibited a characteristic S-shaped striatal ridge in coronal sections. This morphology was dramatically altered in rats with infusions into other areas of the caudate-putamen (Figure 4.10). Medial striatal infusions gave rise to an L-shaped ridge near the cannula infusion site with the vertical part of the "L" along the ventricle and the horizontal part extending orthogonally from the ventricle into the striatum. Infusion into the extreme lateral striatum stimulated the formation of a linear ridge parallel to the wall of the lateral ventricle.

Figure 4.8. Photomicrographs of coronal sections of adult rat brain in an animals receiving nigral 6-OHDA lesion and striatal infusion of TGFα for fourteen days. (A) The striatal ridge was intensely stained by nestin immunohistochemistry. (B) Thionin staining was performed on a near-adjacent section to confirm the registry between the nestin-IR and the striatal ridge.

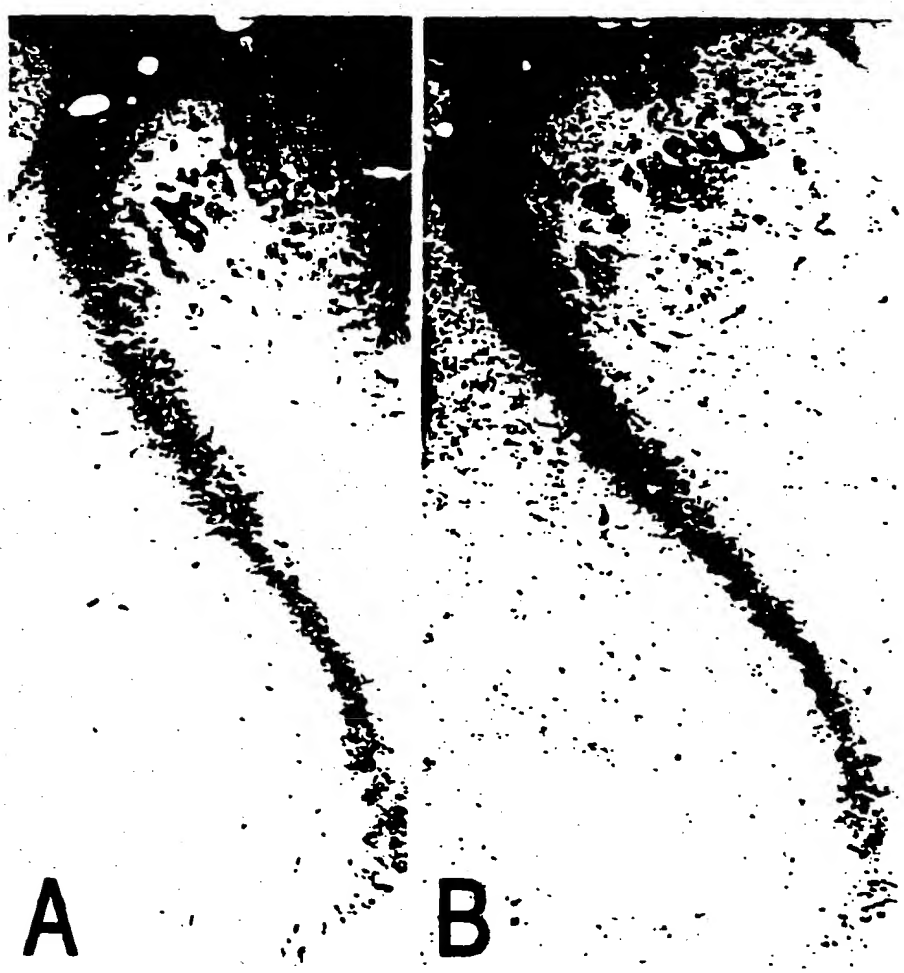

A
B

Figure 4.9. High-power photomicrograph of nestin-IR of a coronal section through the striatum in an animal receiving striatal infusion of TGFα for fourteen days and nigral 6-OHDA lesion. Nestin-IR fibers are primarily oriented orthogonal to the ridge.

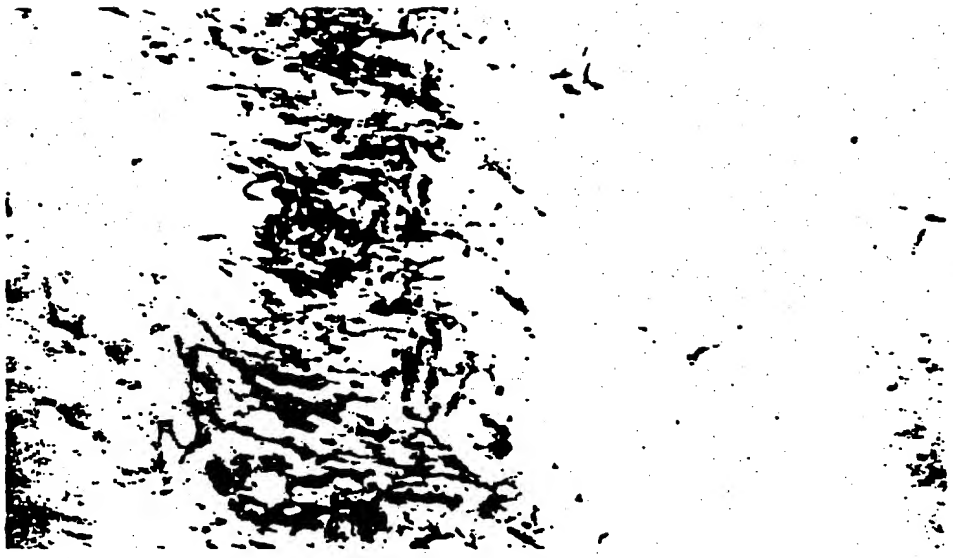
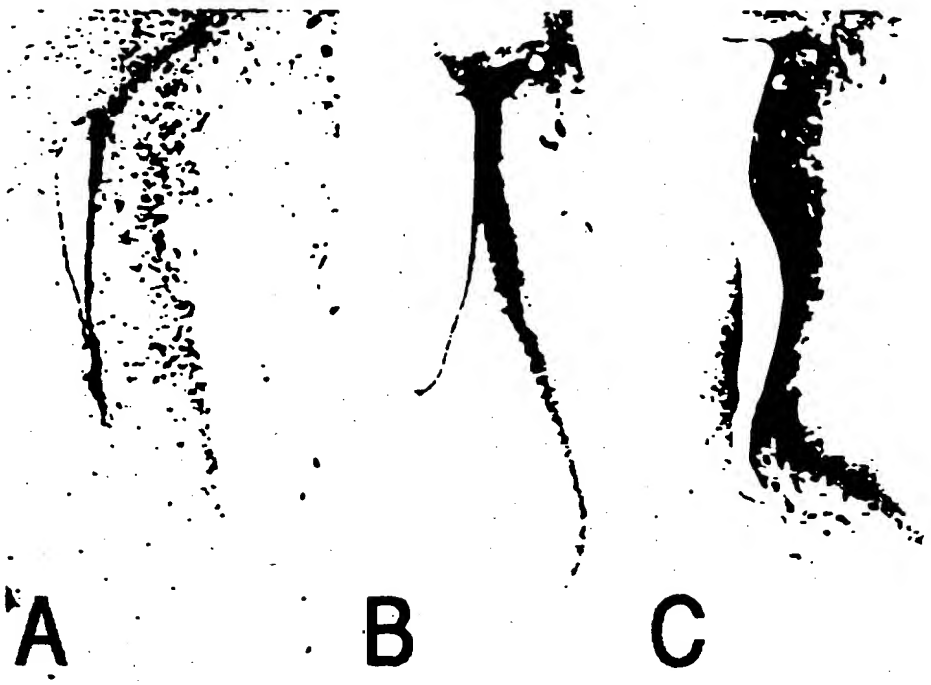


Figure 4.10. Photomicrographs of thionin stained coronal sections from adult rat brains after nigral lesions with 6-OHDA and fourteen-day TGFα intrastriatal infusions at varying distances from the lateral ventricle. In animals where the infusion cannula was implanted in the far-lateral striatum (A), the ridge paralled the subependymal zone and was less dense than with mid-striatal infusions. In animals with mid-striatal infusions (B), the ridge was characteristically S-shaped, with the ventral portion extending far out into the ventral striatum. In animals receiving infusions immediately adjacent to the lateral ventricle (C), the striatal ridge appeared L-shaped and generally exhibited very dense thionin staining.


A
B
C

Vimentin immunohistochemistry. Antiserum recognizing vimentin, a marker for radial glial cells, failed to stain any cells in the striatum, the subependymal zone or the striatal ridge at two weeks of infusion (data not shown).

Control of ridge position. When compared to cells of the ridges in rats used for the *in situ* hybridization experiments, ridge cells in the present series of experiments were maximally displaced much farther from the ventricle (Figure 4.11). The critical difference between these two groups of animals was the timetable of infusions and lesions.

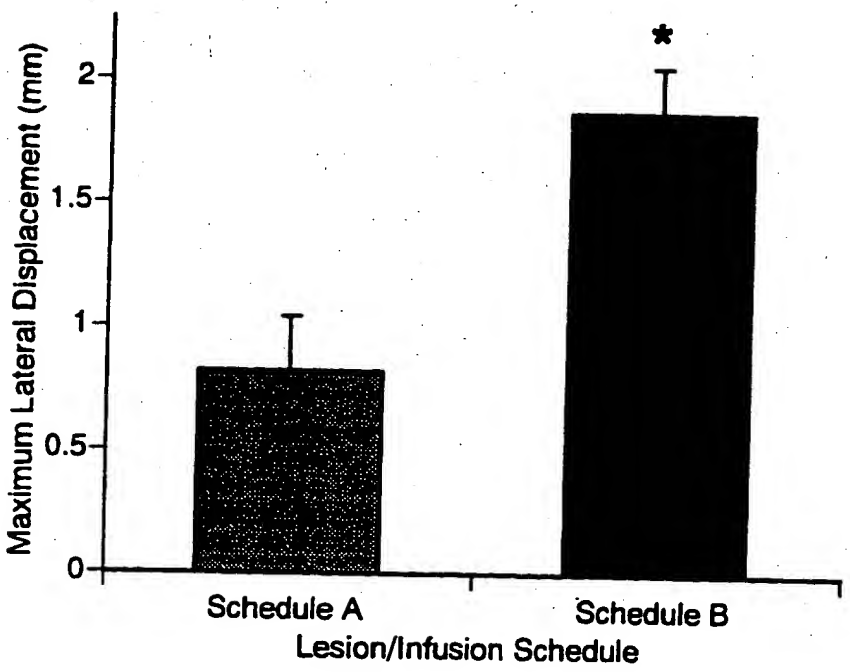
Animals prepared for the *in situ* hybridization experiments received their lesions first. They then underwent a series of behavior tests starting around the second week postlesion to confirm success of the unilateral lesions. Typically, those rats did not receive infusions until five weeks after they had been lesioned: thus, the dopamine degenerative process and the striatal infusion of TGFα were temporally separate events.

In the present series of experiments, the infusions were begun first. Lesions were not performed until 48 hours *after* the infusion pumps were implanted. In these animals, the degeneration of the nigral dopamine neurons, the resulting loss of striatal dopaminergic innervation, and the striatal administration of growth factor were temporally concurrent events.

Infusions into brain regions other than the striatum. All rats receiving infusions into other brain areas received two-week TGFα infusions and nigral 6-OHDA lesions. Intracerebroventricular (ICV) infusion of growth factor ipsilateral to the lesion stimulated the buildup of cells in the adjacent ventricular wall, but did not induce formation of the striatal ridge in any of the animals.

Figure 4.11. Bar graph of maximum displacement normal to the lateral ventricle of cells of the striatal ridge found in coronal sections of adult rat brain after nigral lesion with 6-OHDA and fourteen days of mid-striatal TGFα infusion. Animals receiving lesions first, then TGFα infusions four to five weeks later are included in the "Schedule A" group. Those receiving lesions after the first two days of the 14-day TGFα infusion are included in the "Schedule B" group. Averages ± S.E.M. (Student's *t*-test; P value, * $p < 0.01$).

Maximum Distance of the Striatal Ridge from the Lateral Ventricle in Groups of Rats with Two Different Infusion/Lesion Schedules



Schedule A: 6OHDA lesion first, TGFα infusion weeks later

Schedule B: start TGFα infusion first, lesion 48 hrs after start of infusion

Septal and some striatal infusions stimulated the formation of septal ridges associated with the medial walls of the lateral ventricles (Figure 4.12). Septal ridges, like the striatal ridges, were readily detected with EGF receptor mRNA *in situ* hybridization or with thionin staining, but tended to be qualitatively less robust in terms of the density and number of cells.

Dorsal cortical infusions, that is, infusions so shallow that they did not penetrate the corpus callosum, had no discernable effect on cell density along the lateral ventricle. Neither did these dorsal infusions induce formation of a ridge. Cortical infusions in which the corpus callosum was slightly penetrated stimulated expansion of cells along the ipsilateral ventricle, but did not induce formation of a striatal ridge. These animals did exhibit densities of cells in the corpus callosum that might be considered callosal ridges.

Discussion

The results from the present series of experiments and those from the previous chapter show that TGFα peptide was necessary for the formation of the cellular build-up and the striatal ridge. Not a single animal with a striatal aCSF infusion — whether lesioned or not — displayed any obvious periventricular cellular expansion when compared to subependymal regions contralateral to the infusions or from normal animals. Clearly, cells of the forebrain are responding to striatal infusion of TGFα by increasing their numbers along the lateral ventricle. The obvious question is, "What are these cells and where do they come from?"

Figure 4.12. Photomicrograph of thionin staining of a coronal section from an adult rat receiving TGFα infusion for fourteen days. Septal — and frequently striatal — infusion lead to the formation of thionin-stained ridges in the septum. (is, infusion scar).


is

Recent studies with 6-day ICV or intrastriatal infusions of epidermal growth factor (EGF) in mice demonstrated a large increase in the number of cells around the ventricle immunolabeled with 5-bromo-2'-deoxyuridine (BrdU) or [^{3}H]thymidine, markers for cellular proliferation (Craig et al., 1996). More than ninety-five percent of these cells were also positively immunoreactive for EGF receptor. Cresyl violet Nissl staining also showed an increase in the numbers of cells around the ventricles in these animals in response to EGF administration.

Glial proliferation? The possibility that the expanded cell populations along the lateral ventricles cells were glial cells stimulated by the combined neurotoxic lesion and mechanical injury of the forebrain, and the infused TGFα was considered first. Astrocytes are known to respond to brain injury by proliferating and altering their morphology and functional properties (for review see Norenberg, 1994). Additionally, striatal astrocytes possess EGF receptors (Gómez-Pinilla et al., 1988; Nieto-Sampedro et al., 1988) and are stimulated by TGFα to proliferate (Alexi and Hefti, 1993). In the present study, antiserum against glial fibrillary acidic peptide (GFAP), a marker for astrocytes, failed to demonstrate an increase in astrocytes in the ventricular region or in the ridge at two weeks of infusion. In fact, GFAP-IR was largely excluded from these areas. Normal astrocytic staining was seen medial and lateral to the ridge, for instance, but few GFAP-IR fibers were observed within the ridge itself. This finding paralleled similar findings in experiments with 6-day striatal or ICV infusions of EGF or TGFα: GFAP and an additional marker for astrocytes,

S100ß, showed no significant increase around the lateral ventricle (Craig et al., 1996). Vimentin can also be expressed by reactive astrocytes (Federoff et al., 1984), but vimentin-IR was not observed in any of the sections examined. Markers for microglia (MAC-1) and oligodendrocytes (MAG, CNP, O4 and Rip) also did not change significantly (Craig et al., 1996). Thus, the immunohistochemical evidence demonstrated that the TGFα-induced expansion of cells along the ventricle and in the striatal ridge were not the result of gliosis. Incidentally, an immunomarker for mature neurons NeuN also was not increased (Craig et al., 1996).

Cellular morphology and orientation. Silver and thionin staining clearly revealed the huge numbers of cells comprising the cellular build-up along the ventricle. The cells were densest and most numerous in the dorsal portions of the subependymal zone and ridge. The cells were predominantly fusiform, similar to migrating neuronal progenitor cells in the developing brain, with their long axes oriented orthogonal to the ventricle wall (or to the dorsolateral extension of the subependymal zone bordering the dorsal striatum). Silver stained cells in the ventral segment of the ridge appeared to stream around fiber bundles of the internal capsule, suggesting that they were migrating through the striatum. To determine whether the cells were indeed migrating, a time course experiment was done which examined the development of the ridge and its location as a function of time after the start of the growth factor infusion.

Migration of cells of the striatal ridge. The progressive expansion of cells along the lateral ventricle and the subsequent radial movement of these cells as a dense ridge proved that the cells were indeed migrating *en masse* through the

striatum. As such, the ridge could not have been an anatomical delineation between the rodent putative "caudate-like" and "putamen-like" regions of striatum.

Stimulation of neural precursor cells. Although none of the immunomarkers for mature astrocytes, neurons, microglia, or oligodendrocytes labeled cells of the striatal ridge, monoclonal antibodies recognizing nestin, an intermediate filament expressed by neuroepithelial precursor cells, did intensely stain cell processes in the ridge and along the ventricle. Reactive astrocytes can also express nestin-IR, but the negative GFAP-IR in cells of the ridge eliminated the possibility that astrocytes formed a significant portion of the ridge cells. Nestin-IR is has been used in recent years to identify and label neural precursor cells *in vitro* and *in vivo* (Lendahl et al., 1990; Reynolds and Weiss, 1992; Reynolds et al, 1992; Morshead et al., 1994; Zerlin et al., 1995; Craig et al., 1996). The strong nestin-IR in the striatal ridge and the lack of immunostaining for glial markers provided support for the idea that the cells of the ridge were predominantly neural progenitor cells.

A note on terminology is warranted at this point. Thus far, two distinct cell populations have been identified in adult mammalian brain which can give rise to new neurons and glia (Morshead, et al., 1994). One, the relatively quiescent cell population, are believed to be true multipotent neural stem cells. The other, the constitutively proliferating population, are believed to be neural progenitor cells, descendent from the stem cells. The stem cell population is believed to remain in the ependymal or subependymal zone and replenish the progenitor cell population as they die or migrate away. "Neural precursor" is used here to

describe any of these undifferentiated proliferative cells capable of giving rise to neurons and glia in the adult mammalian brain, whether they be neural stem cells or neural progenitors.

Previous studies have shown that many neural progenitor cells die in the subependymal zone before they can migrate from the region (Morshead and Van der Kooy, 1992). However, it was recently discovered that many others indeed survive and migrate along a highly-restricted path to the olfactory bulbs where they differentiate into olfactory interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994). They migrate tangentially along the wall of the lateral ventricle in a process called "chain migration" wherein chains of migrating cells are ensheathed by specialized GFAP-IR astrocytes (Rousselot et al., 1994; Lois et al., 1996).

The subependymal zone along the forebrain lateral ventricles, then, is much more than a dormant remnant of the embryonic neuroepithelium. In normal unmanipulated brains, it continues to give rise to new neuroblasts that migrate rostrally and differentiate into neurons. Under the influence of EGF-family neurotrophic factors, including TGFα, subependymal neural precursors can be stimulated *in vitro* to give rise to large numbers of new neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992). From these explant studies, it became clear that the highest concentrations of neural precursor cells were found in the dorsal portion of the subependymal zone, along the dorsal border of the caudate-putamen.

There is even some recent evidence that neural precursors may be stimulated to increase their numbers and produce new neurons and glia *in vivo*

(Craig et al., 1996). Cells double-labeled with BrdU and markers for mature neurons and glia were found diffusely distributed throughout the striatum, septum and cortex after six days of ICV infusion of EGF and up to seven weeks of post-infusion survival. No mass migration of subependymal cells into the adjacent striatum was observed in that study and the numbers of cells were quite modest compared to the densely-packed mass of cells observed in the striatal ridge in our experiments. None of the animals in that study received the longer infusions (at least nine days in our studies) necessary to stimulate the mass migration and none of the animals received nigral 6-OHDA lesions which dramatically increased the incidence of migration.

Evidence of their identity. A remaining question is whether the cells comprising the massive expansion along the ventricle and the migrating striatal ridge truly were neural progenitor cells. Table 4.2 summarizes the data supporting the conclusion that these cells were indeed neural progenitors. The neurochemical evidence showed that they do not express markers for mature neurons, astrocytes, oligodendrocytes or microglia. They did, however, intensely express a marker for immature neural progenitor cells. They expressed markers for cellular proliferation. They arose from the wall of the lateral ventricle where neural precursors are located in the adult rodent brain, expanded laterally and migrated radially away from the ventricle. Furthermore, their cellular morphology was fusiform and their processes were oriented orthogonal to the ventricle and the ridge, similar to migrating neural progenitors in the embryonic brain and consistent with their migration from the subependymal region. They expressed abundant EGF receptor mRNA and EGF

Table 4.2. Summary of the experimental evidence from the present studies and those of Craig et al., 1996, suggesting that cells of the periventricular expansion and the striatal ridge are neural progenitors arising from subependymal neural stem cells. (*denotes neurochemical markers included as part of the present studies).

Evidence of Progenitor Phenotype for Cells of the Expansion and Ridge

Neurochemical

Express abundant EGF receptor mRNA* and immunoreactivity

Immunonegative for GFAP* or S-100ß, markers for astrocytes

Immunonegative for NeuN, a marker for mature neurons

Immunonegative for MAG, CNP, O4, or Rip, markers for oligodendrocytes

Immunonegative for vimentin*, a marker for radial glia

Immunonegative for MAC-1, a marker for microglia

Immunopositive for nestin*, a marker for neuroepithelial precursors

Morphological

Elongated somata in ridge oriented orthogonal to subependymal zone

Nestin-IR processes in ridge oriented normal to subependymal zone

Anatomical

Arise from the subependymal zone

Highest density of cells is in the dorsal subependymal region

Physiological

Respond to TGFα administration by increasing their numbers

Ninety-five percent overlap of EGF-IR and BrdU-labeled cell populations

receptor immunoreactivity and proliferated in response to EGF-family neurotrophic factors as subependymal neural precursors can be stimulated to do *in vitro*. Moreover, the numbers of cells were greatest in the dorsal subependymal zone where the largest numbers of neural precursors are located. Taken together, the evidence overwhelmingly supports the view that these cells are indeed neural progenitors.

Mechanisms of migration. The mass migration of these cells into the striatum was influenced by striatal dopamine denervation and by the location of the infusion cannula, but the mechanism of migration was unclear. The fact that the shape of the ridge could be modified simply by changing the site of infusion initially suggested a chemoattractant effect. TGFα is known to be a potent chemoattractive agent for diverse cell types (Ju et al., 1993; Panagakos, 1994). Its abundant expression in the perinatal caudate-putamen may indicate that it performs a similar role in the development of the striatum.

Neural precursor cells in the normal brain are located in a thin region in the wall of the lateral ventricle. Infusions into the mid-striatum were closer to cells of the dorsal end of this region. Presumably, cells migrating into the striatum would move toward putative higher concentrations of growth factor at the tip of the infusion cannula where the TGFα was released. The characteristic S-shape of ridges in animals with mid-striatal infusions might have resulted from receptor saturation of cells in the dorsal segment of the subependymal zone nearest the tip of the infusion cannula. Cells with saturated EGF receptors might have halted their migration once they moved close to the infusion site. Cells near the ventral end of the subependymal zone would see a lower putative

growth factor concentration and would have to travel farther toward the infusion site before the concentration of TGFα increased enough to saturate their receptors. This differential migration with receptor saturation might explain the characteristic S-shape of these ridges.

Infusions into the medial striatum resulted in L-shaped ridges, again in keeping with a neurochemical gradient/receptor saturation effect. In this instance, dorsal subependymal cells may have had their receptors saturated and their migration halted before they could even emerge from subependymal zone. Only cells in the most ventral portion of the subependymal region could migrate away from the ventricle.

Extreme lateral infusions essentially would have been presented a similar putative concentration gradient to cells along most of the length of the proliferative region. The subependymal cells all migrated a similar distance from the ventricle, resulting in a roughly linear ridge, consistent with the idea of a chemoattractant, neurochemical gradient effect.

However, immunohistochemical evidence from the characterization studies cast doubt on the idea that a simple chemoattractant effect could entirely explain the mass radial migration. Nestin-IR processes of the migrating cells were not aligned with the tip of the infusion cannula, the region of the putative highest concentration of growth factor. Instead, they were oriented normal to the ventricle and the ridge. This orientation suggested that the cells migrated orthogonally into the caudate-putamen — as migrating neural progenitors do from the embryonic striatal neuroepithelium — not obliquely toward the tip of the infusion cannula.

Two additional findings discounted the role of simple chemoattraction in the migration of the ridge. First, the cells did not begin to migrate as they were produced; they increased their numbers along the ventricle for a period of more than a week, then migrated *en masse* as a dense sheet of cells. Further, lesion of the ipsilateral substantia nigra greatly increased the incidence of migration. Both of these observations suggested a more complex set of factors influencing the migration of the cells. These data did not entirely rule out a role for chemoattraction in the migration of the ridge, but they did indicate that a simple chemoattractive effect could not by itself account for it.

Another mechanism that might have contributed to the radial migration of neural progenitors in the adult striatum was the reconstruction of the radial glial scaffold due to the neurotoxic lesion, the mechanical injury done by the surgical implantation of the infusion cannula, or both. Radial glia guide the migration of neural progenitor cells in many regions of developing brain. They are anchored along the ventricle and extend their processes radially into the overlying parenchyma. They normally are transformed into GFAP-IR astrocytes in the early postnatal period once neuroblast migration is complete, and cease expression of vimentin and nestin.

Freezing injury of the cortical plate in neonatal rats inhibited radial glial transformation and caused the persistence of glial expression of vimentin and nestin in the injured regions of the adult brain (Rosen et al., 1994). Kainate lesion of the adult rat hippocampus induced radial glial morphology and expression of nestin-IR and vimentin-IR in astrocytes of the hippocampal

subependymal zone, suggesting that brain injury could stimulate a reversion of astrocytic phenotype to one found in the embryonic brain (Clarke et al., 1994).

Data from our immunohistochemical characterization experiments, however, do not provide support for this phenomenon in the present study. Nestin-IR fibers were found in abundance in radially-oriented fibers along the ventricle at infusion day nine, but at later time points, they no longer remained along the ventricle. As the cells of the ridge migrated away from the subependymal region, so did the nestin-IR fibers. Furthermore, immunostaining for vimentin, a specific marker for radial glia, did not reveal any labeled fibers either in the ridge or in the subependymal region. Thus, it is unlikely that any astrocytes were reverted to their embryonic radial glial phenotypes or that astrocytic reversion played a role in the radial migration of the neural progenitor cells.

A newly-described mode of migration employed specifically by neural progenitors in the adult mammalian brain elucidates the tangential movement of these cells from the forebrain subependymal zones to the olfactory bulbs (Lois et al., 1996). Rostrally migrating neuroblasts are densely packed and sheathed by GFAP-IR astrocytes bordering their highly-restricted migratory pathway. The migrating cells essentially form a solid stream of moving cells within a tube of specialized glial guide cells. The neural precursors in our experiments migrated as a sheet through the striatal neuropil — not along a restricted path — and were not associated with GFAP-IR cells. In fact, the proliferative subependymal zone and the cellular ridge largely excluded GFAP-IR. Thus, the

mechanism of chain migration could not account for the radial migration seen in the present studies.

Another mechanism possibly underlying the mass neural progenitor migration was that cells of the striatum may have altered their expression of growth factors, cell adhesion molecules, or other substances in response to injury. In this scenario, the striatum may have been stimulated to provide its own chemoattractants or molecules that facilitate radial migration. Alternatively, it may also have been induced to downregulate expression of substances that inhibit migration.

Recent studies examining cell adhesion molecules in the striatum and subependymal region provide particularly intriguing insight. Highly polysialylated neural cell adhesion molecule (PSA-N-CAM) immunoreactivity is intensely expressed in the developing rodent striatum, but decreases as the animal matures (Aaron and Chesselet, 1989; Szele et al., 1994). PSA-N-CAM expression, however, persists along the adult forebrain subependymal region (Rousselot et al., 1994; Szele et al., 1994). Partial decortication-induced striatal deafferentation dramatically increased expression of PSA-N-CAM and another adhesion molecule, L1, in the subependymal zone (Poltorak et al., 1993; Szele and Chesselet, 1996). In the human brain, PSA-N-CAM expression is low in normal striatum, but is increased in the striata of Huntington's disease patients, particularly in the subependymal zone (Nihei and Kowall, 1992).

Of special interest were the changes occurring in fibronectin mRNA expression in the striatum after partial unilateral frontal decortication (Popa-Wagner et al., 1992). Fibronectin is one of a number of molecules that have

been shown to support neural migration *in vitro* (Fishman and Hatten, 1993). Fibronectin mRNA hybridization was increased to a maximun level at 72 hours in the portion of the striatum immediately under the wound cavity. This early increase was interpreted as a component of the short-term wound healing process. Fibronectin expression in the greater ipsilateral striatum followed a longer-term increase, peaking at about ten days post lesion. This secondary increase was interpreted as the striatal response to deafferentation. Increases in expression of two other mRNAs that code for N-CAM and alpha tubulin followed only the early wound healing-related spatial and temporal patterns.

The ten-day peak of striatal fibronectin mRNA expression after deafferentation corresponds well to the delay of ridge migration following striatal dopamine denervation in our studies. The delay of peak fibronectin mRNA expression may help explain why progenitor cells in our studies did not begin to migrate radially until around the ninth day of infusion, and why, when they finally did migrate, they migrated *en masse*. In animals where the infusions and lesions were separated by several weeks, the maximum lateral migration distances at two weeks of infusion were dramatically reduced. This observation, too, is consistent with the transient peak in striatal fibronectin expression. In the few animals with ridges that did not also receive nigral lesions, mechanical injury of the overlying cortex may have stimulated enough fibronectin expression in the striatum to facilitate the migration. Fibronectin, then, may be upregulated in response to dopamine denervation of the ipsilateral striatum and, in turn, may temporarily facilitate radial migration of neural progenitors from the subependymal zone.

Another possible influence on radial migration of neural progenitors in the adult striatum may stem from a secondary effect by cell adhesion molecules. Infusion of N-CAM into the brains of adult rats receiving stab wounds to various areas of the brain, including striatum, inhibited astrocytic proliferation (Krushel et al., 1995). Astrocytes release factors inhibiting neurite outgrowth and may thus inhibit neural regenerative responses. Thus, denervation of the striatum, and the associated increase in subependymal PSA-N-CAM may release inhibition of neural regeneration.

The enhancement of the migration effect in dopamine-denervated striatum may also have been related directly to the loss of dopaminergic innervation. During embryonic development of the striatum, immature neurons originate in the ventricular region and migrate radially (Bayer, 1984; Bayer and Altman, 1987). The developmental migration of striatal neurons takes place prior to dopamine innervation by afferents from the midbrain. Stimulation of D_2 dopamine receptors on hypothalamic neurons dramatically attenuated TGFα mRNA expression and pituitary growth (Borgundvaag et al., 1992). Although dopamine receptor-mediated inhibition of TGFα expression has not been studied in the subependymal zone, it is consistent with the depressed incidence of migration in non-lesioned animals. Thus, dopamine innervation during development may inhibit migration of striatal cells as the forebrain dopaminergic innervation becomes established.

Striatal dopamine may also contribute to the downregulation of striatal TGFα early in postnatal development as dopaminergic afferents become

established. Dopamine denervation of the adult striatum may mimic for striatal cells some of the local chemical environmental cues normally only seen in the developing striatum — for instance, a reduction of available ligand for dopamine receptors expressed on striatal neurons. Striatal dopamine innervation has also been linked in a reciprocal manner to expression of extracellular matrix (ECM) molecules by astrocytes (Gates et al., 1993). Interestingly, TGFα is selectively elevated in the striata of PD patients (Mogi et al., 1994) similar to the elevated expression in the embryonic striatum. If striatal TGFα is regulated by dopamine innervation, this increase may relate to the reduction of striatal dopamine and the consequent release of inhibition of TGFα expression.

Summary. Whatever the underlying mechanism, the time course experiment proved that subependymal cells could be stimulated to increase their numbers and migrate radially *en masse* into the adjacent striatum in the adult rat brain. Experiments in which the location or the dose of TGFα infusion was varied showed that movement of the striatal ridge and the gross numbers of cells involved could be controlled. The characterization experiments provided abundant evidence that the subependymal cellular expansion and the dense striatal ridge were composed of neural progenitor cells. In the next chapter, the importance of these discoveries is discussed along with their potential application for the treatment of human neurodegenerative disease and traumatic brain injury.

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CHAPTER FIVE

CONCLUSIONS AND DISCUSSION OF POTENTIAL APPLICATION FOR THE TREATMENT OF NEURODEGENERATIVE DISEASE

In the previous chapter, evidence was presented that neural precursors in the adult rodent forebrain subependymal zone could be stimulated to greatly expand their numbers and migrate radially *en masse* into the striatum in response to intrastriatal infusion of TGFα. Furthermore, the incidence of their migration as a dense ridge of cells could be increased by striatal dopamine deafferentation with nigral injection of 6-OHDA.

The shape of the striatal ridge could be altered by adjusting the infusion site. Similarly, the gross numbers of cells comprising the ridge could be manipulated by the dose of TGFα or the proximity of its release relative to the population of neural stem cells in the subependymal region. In animals receiving mid-striatal or medial striatal infusions, the migrating cells stopped before they reached the infusion cannulae, resulting in S- or L-shaped ridges. Thus, it may be possible not only to facilitate the cells' migration, but to control where they halt their migration as well. Adjusting the dose and the location of release of the growth factor (and perhaps other compounds) may allow restriction of the area affected to a relatively limited target region.

It is clear that the proliferation and migration of neural precursor cells in the adult mammalian brain are distinct and separately-controllable events. ICV or intrastriatal infusions of TGFα or EGF without deafferentation can induce

the former, but neurochemical or mechanical (or chronic neurodegenerative) deafferentation may be necessary to facilitate the latter, at least on a large scale. It may even be possible to mimic the facillitory effect of deafferentation with pharmacological agents. For instance, if transient striatal expression of cell adhesion molecules is indeed key to supporting the migration, it may be stimulated by the co-administration of additional inductive compounds with the growth factor. Fibronectin, for instance, can be strongly upregulated by transforming growth factor beta (TGFß) in cultures of cerebellar astrocytes (Baghdassarian et al., 1993). In transgenic mice overexpressing TGFß, fibronectin and laminin are also strongly increased in the CNS over normal levels (Wyss-Coray et al., 1995).

Forebrain neural stem cells which give rise to the migrating progenitors are believed to remain in place along the ventricular wall (Morshead et al., 1994). In the experiments presented in chapters five and six, a region of intense EGF receptor hybridization persisted along the lateral ventricle after the migratory ridge had moved into the striatum. In addition, elongated cells were always found between the ridge and the lateral ventricle. Thus, despite the mass cellular migration away from the subependymal zone, the stem cells themselves likely were not part of the migrating ridge. These neural stem cells may provide a renewable source of new neurons and glia. It may be possible, then, to stimulate migration of multiple waves of neural progenitor cells into injured or degenerated brain regions. It also suggests that the normal function of stem cells in the adult forebrain — to provide new neurons for the olfactory bulbs — may not be irreversibly disrupted by the treatments.

Interestingly, the proliferation and radial migration stimulated by the growth factor infusions and nigral lesions in the present experiments may themselves be part of a process occurring normally in the adult mammalian brain. The two primary stimulants of neural stem cell proliferation and migration in the present studies, namely increased striatal TGFα and dopamine deafferentation, are present to some extent in the brains of PD patients (Mogi et al., 1994; Javoy-Agid et al., 1984). We might expect low level proliferation and radial migration in response to these cues in the degenerating parkinsonian brain. The striatal administration of a large dose of TGFα and the acute dopamine deafferentation with 6-OHDA lesion, may then enhance a similar process already occurring in the diseased or injured adult brain.

It should be noted that abundant striatal expression of TGFα (and its mRNA) and a lack of dopaminergic innervation also characterize the early developing striatum (Weickert and Blum, 1995; Bayer, 1984). Similarly, the increased EGF receptor mRNA abundance in the subependymal region in the TGFα-infused animals mimics the abundant EGF receptor mRNA hybridization observed in the periventricular neuroepithelium in the developing brain (see Appendix for photomicrographs; Seroogy et al., 1994; Seroogy et al., 1995). Messenger RNAs encoding forms of fibronectin, and its receptor, and other cell adhesion molecules which may facilitate the migration of neural precursors, are also developmentally regulated (Pesheva et al., 1988; Prieto et al., 1990; Pagani et al., 1991; Linnemann et al., 1993). Thus, the effects observed in the TGFα-infused and 6-OHDA lesioned animals in the present studies may represent a

selective recapitulation of embryonic neurogenesis. That is, neural stem cells in the adult mammalian brain may respond to chemical proliferation and migration signals as they do in the developing animal.

Neural stem cells have very recently been found in subependyma throughout the adult rodent CNS (Weiss, 1995; Ray et al., 1996) and in the subependyma of the adult human forebrain (Kirschenbaum et al., 1994). Manipulation of these cells potentially could provide a source of new neurons for diseased and injured CNS tissue in diverse regions of the brain and spinal cord. One of the techniques proposed for utilizing these cells first involves removal of neural stem cells from a patient with a neurodegenerative disorder. The cells would then be grown *in vitro* to generate large numbers of neural progenitors, then re-implanted into the same patient (Stein et al., 1995). This technique has some advantages over the use of embryonic cells from aborted fetuses such as obviation of the need for aborted fetuses as tissue donors and the absence of a tissue rejection response.

Stimulating proliferation and migration of neural precursors *in vivo* shares these advantages over fetal cell grafts with the culture technique; however, it has substantial additional advantages over either of these strategies. *In vivo* stimulation could reduce the extent and possibly the number of invasive neurosurgical procedures. No stem cell excision surgery would be performed and multiple plugs of transplanted cells — typical of embryonic or cultured cell grafts — would not be necessary. In addition, there would be no massive die-off of undifferentiated neural progenitor cells due to the transplantation procedure. With human fetal dopaminergic cell grafts, typically 90% to over

99% of the implanted cells die before they can become established in the host brain (Freed et al., 1996).

Another advantage is that neural progenitor cells would not be isolated from the host brain by scar tissue. Plugs of transplanted cells become encapsulated within an envelope of glyotic scar tissue and reactive astrocytes. In addition to the physical barrier of the dense glyotic tissue, reactive astocytes within the scar tissue release factors which inhibit neurite outgrowth (McKeon et al., 1995;). Neural progenitors created *in vivo* are not isolated from the rest of the brain by scar tissue. The outgrowth of their neurites, therefore, would not be inhibited by a massive proliferation of reactive astrocytes.

The technique presented here also represents an advance over the single previous study of forebrain neural stem cells stimulated *in vivo*. In that study, adult rats received ICV infusions of EGF for six days and were followed for up to seven weeks post-infusion (Craig et al., 1996). In the present study, TGFα was infused for fourteen days and followed for up to three months following the end of infusion. Only in the present study did the cells of the periventricular expansion migrate *en masse* into the overlying striatum. The directed mass migration of neural progenitors into a selected target area may represent a preferred method to repopulate degenerated brain regions with new neurons.

The primary focus of the present work is the characterization of the cellular expansion and mass migration. Differentiation of the cells was not examined. It is likely that some of the cells derived from the striatal ridge spontaneously differentiated if they behaved like the progenitor cells in the other *in vivo* study (Craig et al., 1996). One area of intense recent interest is the manipulation of

neural stem cell differentiation. Both the final location and the neurochemical phenotypes of the cells once they have differentiated are of primary importance.

When neural precursor cells were removed from adult rodent brains and differentiated *in vitro*, cells immunochemically identified as astrocytes, oligodendrocytes and neurons are seen (Reynolds and Weiss, 1992; Reynolds et al., 1992; Lois and Alvarez-Buylla, 1993). Many of the cells identified as neurons also expressed immunoreactivity for GABA and substance P, neurochemical markers for two cell types normally found in the striatum. Precursor cells explanted from the adult human brain also expressed neuronal markers and displayed electrophysiological properties associated with neurons (Kirschenbaum et al., 1994).

These experiments suggest that when cells of the striatal ridge spontaneously differentiate *in vivo*, many of them will become cells with phenotypes typical of striatal neurons. Some very recent data suggests that their phenotypes can be altered by exposure to different combinations of neurotrophins (Lachyankar et al., 1996). Progenitor cells receiving different treatments expressed different neurochemical immunomarkers once they differentiated, including acetylcholinesterase, GABA, tyrosine hydroxylase (TH) and calbindin. The expression of TH was particularly interesting, since combined proliferation, migration and directed differentiation into dopamine cells could provide a novel method to replace striatal dopamine lost in Parkinson's disease (PD).

In PD patients, functionally significant numbers of new dopamine-producing striatal cells might aid in the reversal of motor deficits in a manner

similar to transplants of aborted fetal midbrain tissue. In patients with Huntington's disease (HD), neural precursors might be stimulated to repopulate the striatum with new medium spiny GABAergic neurons lost to the disease. Some recent evidence from a different line of research indicates that reconstruction of the striatopallidal pathway itself might be possible. Conditionally immortalized neural progenitor cells transplanted into the striatum differentiated and sent processes from the striatum to the globus pallidus (Lundberg et al., 1996).

Clearly, the research of neural stem cells in adult mammalian brains is itself in the embryonic stage. It will be fascinating to see whether these cells can be stimulated to repopulate injured brain regions and actually reconstruct pathways lost to injury or disease. As a final note, it is perhaps ironic that one of the prominent emerging strategies for arresting or reversing the devastating effects of neurodegenerative disease is the transplantation of undifferentiated cells from aborted human fetuses or even fetuses of other species: the mature brain may be able essentially to provide its own graft if conditions similar to those in the developing brain are reproduced.

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APPENDIX

EXPRESSION OF TGFα AND EGF RECEPTOR mRNAS IN THE NORMAL DEVELOPING AND ADULT NIGROSTRIATAL SYSTEM

Introduction

As reviewed in Chapter Two, mRNAs encoding EGF-family neurotrophic factors are developmentally regulated in the nigrostriatal system. Here, the expression of TGFα and EGF receptor mRNAs are examined in the normal developing and adult rodent system. Not all developmental stages are represented by more than two animals, so the usual statistical comparisons cannot be made. However, as complete series of sections across all studied ages were hybridized and apposed to autoradiographic film together, the marked changes in distribution and abundance occurring with nigrostriatal maturation can nevertheless be appreciated.

Materials and Methods

Animals and tissue preparation. Adult male adult and timed-pregnant female Sprague-Dawley rats (250-350 g) were obtained from Simonsen (Gilroy, CA). The animals were maintained in a temperature and humidity controlled campus vivarium. Use of the animals and the experimental procedures employed were approved by the University of California, Irvine, Animal Research Committee in accordance with National Institutes of Health guidelines.

Tissue preparation. Newborn (P0), postnatal day 1 (P1), and P4 animals animals were anesthetized by hypothermia and sacrificed by decapitation. P10, P21 and adult animals were sacrificed by decapitation. Their brains were quickly removed, frozen in isopentane at -20°C and stored at -70°C. Coronal cryostat sections were cut at 20 μm and thaw-adhered to Vectabond (Vector Labs, Inc.) coated slides in ordered anterior-to-posterior rows. The sections were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, rinsed in phosphate buffer and air dried. Sections were stored with desiccant at -20°C until processed.

Hybridization probes. TGFα mRNA probes were generated from a 550 nucleotide XbaI/BamHI cDNA fragment from the 5' end of rat TGFα (kindly provided by Drs. M.S. Kobrin and J.M. Korc, University of California, Irvine) subcloned into pGEM 7Zf (Promega, Inc.). Antisense and sense probes were transcribed with SP6 and T7 polymerases, respectively. Rat EGF receptor mRNA probes were produced from a 718 base pair BamHI/SphI insert from the 5' end of the gene (also courtesy Drs. M.S. Kobrin and J.M. Korc, U.C. Irvine) in pGEM 7Zf. Probes for rat TH were created using the 1.2 kb BamHI/EcoRI fragment subcloned into pGEM 7Zf. Antisense subclones for EGF receptor and TH were transcribed with T7 polymerase. Sense subclones for EGF receptor and TH were transcribed with SP6 polymerase. All probes were radiolabeled by transcription in the presence of [^{35}S]UTP (NEN Research Products, Inc.).

In situ *hybridization and analysis. In situ* nucleic acid hybridization was performed according to the method described by Simmons et al., 1989 except

that developing brains were treated with 0.0001% proteinase K solution and 0.05 M EDTA. Sections were hybridized overnight at 65°C with sense or antisense probes at a concentration of 10^7 c.p.m./ml. Adjacent sections from the same animals were hybridized to each of the probes so that direct comparisons could be made of their anatomical distributions.

Slides from developing and adult animals were grouped together and apposed with ^{14}C-labeled brain paste standards to autoradiographic BetaMax Hyperfilm (Amersham, Inc.) for six to seven days. After successful development of the autoradiography film, the slides were dipped in Kodak NTB-2 emulsion and exposed for four weeks. The autoradiographic sheet film and NTB-2 emulsion were developed with D-19 developer and Rapid Fix (Kodak, Inc.). The brain sections were then counterstained with thionin and coverslipped. Dipped and stained sections were examined semiquantitatively and photographed under bright and dark field microscopy.

Results

Expression of TGFα and EGF receptor mRNAs in the nigrostriatal system was traced through selected time points from early postnatal development to adulthood. TGFα mRNA hybridization was found in abundance in the early postnatal striatum but was gradually reduced to near adult levels by P21 (Figure A.1). Expression in the corpus callosum increased through postnatal development to levels comparable to those in the striatum (Figure A.1). TGFα

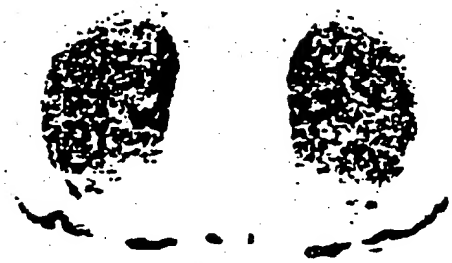
Figure A.1. Photomicrographs of autoradiograms of coronal rat forebrain sections from animals of early postnatal to adult stages of development showing striatal expression of TGFα mRNA. (A) On the day of birth (postnatal day 0, P0), hybridization in the striatum was most intense. The gradually decreasing level of striatal expression with maturation was traced through probed sections from P1 (B), P4 (C), P10 (D), P21 (E) and adult (F) animals. In contrast to the decreasing striatal expression, TGFα mRNA abundance progressively increased in the corpus callosum (cc).

A

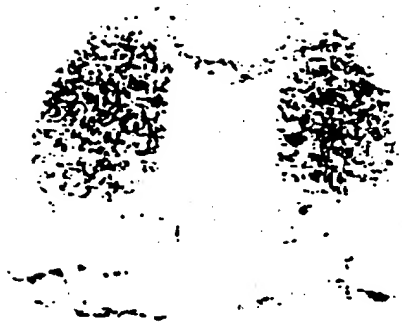
B

C

D



E



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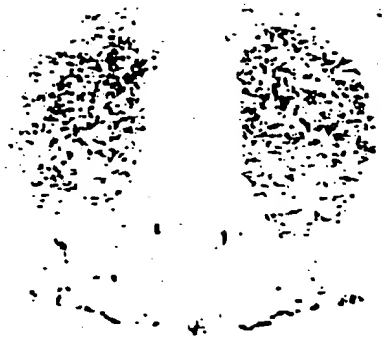

mRNA was not detected in significant abundance in the developing or the adult substantia nigra (data not shown).

Striatal EGF receptor mRNA peaked early in postnatal development and decreased again by P21 (Figure A.2). EGF receptor was highest in the neuroepithelia around the lateral ventricles, but was also found at moderate levels in the body of the striatum. In the developing ventral midbrain, EGF receptor mRNA was barely detectable in early postnatal brains, but gradually increased to moderate levels by P21 (Figure A.3).

In adult animals, TGFα mRNA expression was moderate in the striatum and low-to-moderate in the ventral striatum and nucleus accumbens (Figure A.1). EGF receptor mRNA hybridization was found at low levels in the body of the striatum and nucleus accumbens with higher punctate expression dispersed throughout (Figure A.2). It persisted at moderate levels in the regions of striatum immediately bordering the lateral ventricles (Figure A.2).

In the adult ventral midbrain, EGF receptor mRNA hybridization was found in the substantia nigra (SN), particularly the medial pars compacta, and the paranigral and parabranchial nuclei of the ventral tegmental area (VTA) (Figure A.3).

Discussion

Previous studies indicated that TGFα and EGF receptor mRNAs are strongly regulated during ontogeny of the nigrostriatal system and that their expression in the adult largely represents a continuation of the developmental pattern (Lazar and Blum, 1992; Loughlin et al., 1994; Seroogy et al., 1994;

Figure A.2. Photomicrographs of autoradiograms of coronal rat forebrain sections from animals of early postnatal to adult stages of development showing striatal expression of EGF receptor RNA. At P0 (A) and P1 (B), hybridization was moderate in the striatum and slightly higher in the neuroepithelia surrounding the forebrain lateral ventricles. Expression peaked at about P4 (C), dropped markedly by P10 (D) and continued to decrease to levels similar to those in mature animals by P21 (E). EGF receptor mRNA hybridization in the adult (F) was moderate immediately along the lateral ventricles and low in the body of the striatum with punctate expression at higher abundance.

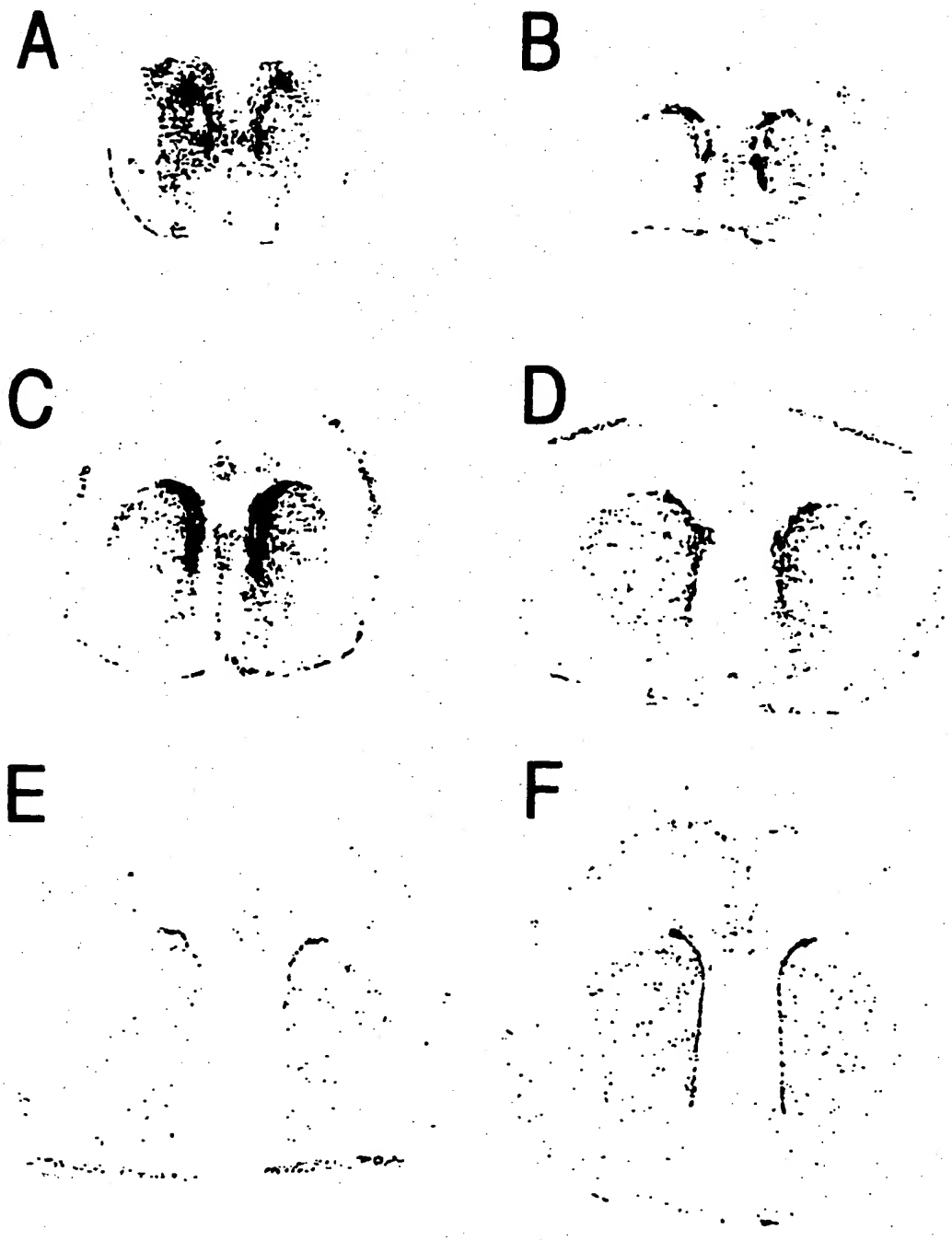
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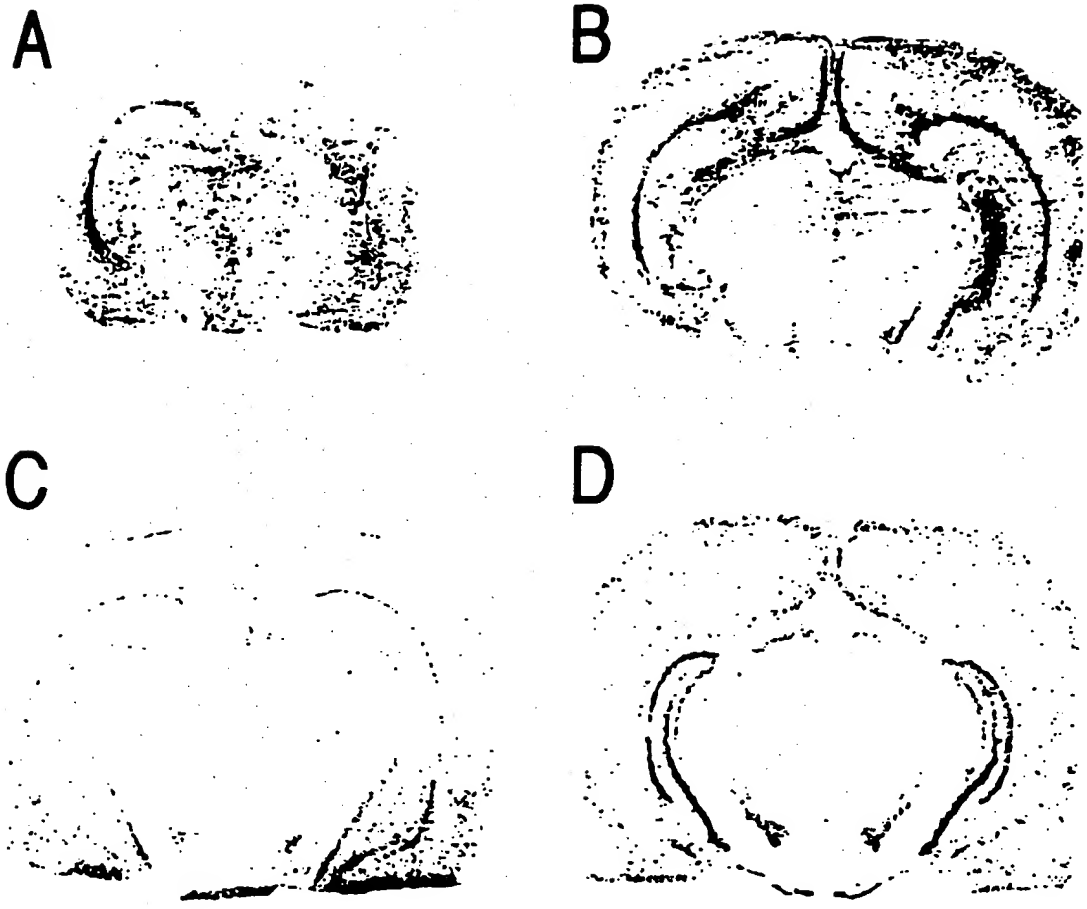
Figure 4.3. Photomicrographs of autoradiograms of coronal rat midbrain sections from animals of postnatal to adult stages of development showing mesencephalic hybridization to EGF receptor RNA. At P4 (A) and earlier timepoints, TGFα mRNA was not detectable over background in the ventral midbrain. At P10 (B), P21 (C) and adult (D) stages, EGF receptor mRNA was expressed at low-to-moderate abundance in the substantia nigra (sn), particularly the medial portion, and adjacent nuclei of the ventral tegmental area (vta).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Seroogy et al., 1995; Weickert and Blum, 1995). TGFα and EGF receptor mRNA hybridization in our developing and adult animals closely paralleled the findings of these earlier reports. The persistence of their expression in the adult striatum and midbrain is consistent with a supportive role in the mature nigrostriatal system.

The moderate EGF receptor mRNA expression in the adult subependymal regions along the forebrain lateral ventricles suggests a role in the maintenance or function of cells in this region as well (Seroogy et al., 1995; Weickert and Blum, 1995). TGFα (or EGF) has been shown to support the survival and differentiation of "EGF-responsive" cells from this region when they are explanted and grown *in vitro* (Reynolds and Weiss, 1992; Reynolds et al., 1992). It may perform a similar function *in vivo* during development. Also, striatal TGFα expression may play a role in the radial migration of cells from the forebrain neuroepithelium in the normal developing striatum (Bayer, 1984).

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